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RESIDUE EVALUATION OF VETERINARY ANTIBIOTIC BY HPLC METHOD IN MEAT OF BROILER AND INDIGENOUS CHICKEN OF RURAL AREAS IN QUETTA, PAKISTAN

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ABSTRACT

Antibiotics are broadly used in the poultry industry globally. Many antimicrobial agents have been practiced for a long to protect the animal's health and enhance their productivity. These agents may often be added to the animal's food as growth promoters. Food-producing animals are the main source of protein; unfortunately, edible meat has a high concentration of drug residues which may cause detrimental effects on consumer's health. To investigate the presence of veterinary drugs in food items such as chicken meat, the present study was conducted with the main aim to evaluate the residual concentration of four universally used antibiotics in muscle and liver samples of broilers. The quantitative detection for antibiotics was performed by using the method of HPLC. The obtained results showed the misuse of drugs or lack of application of antibiotics in the recommended withdrawal period. Samples that contained residue levels of Amoxicillin, Oxytetracycline, Enrofloxacin, and Tylosin higher than maximum residues limits (MRLs) were 80% in broiler and 20% in indigenous, while some samples were below the permissible limits. This contamination in chicken meat needs to develop legislation about residue levels before marketing in Balochistan. As well as the regular inspection of poultry farms is necessary to inform the farmers about the hazardous effects of antibiotics on human health.

KEYWORDS: Detection, License, Localization, Recognition and Segmentation.

1. INTRODUCTION

Numerous types of antibiotics are utilizing for increasing the production of broiler and their meat (Sarker et al., 2016). The poultry industry is considered one of the fastest and largest rising agro-industries among others and it is the second most widely used meat (Johnston, 2001; Mahgoub et al., 2006). The major reason for residues found in animals is possibly the cross-contamination such as in the feed mills the contaminated feedstuffs being used. These

drugs residues and their metabolites after administration caused resistance because it remains for a longer time in the body of consumers. These residues are highly detected in organs that show higher values and exceed the MRLs (maximum residues limits) such as kidney, liver, and fat body (storage tissues) as they metabolize and secrete them. Also present in bones and muscles of poultry and livestock. Their concentration is also varying in different tissues of chicken and consumers. (Abedullah & Bukhsh, 2007).

The primary source of animal protein in Pakistan is poultry chicken meat (Abdullah & Bukhsh, 2007). It was estimated that the poultry chicken production was developing significantly per year in Pakistan. The meat production was 1074 thousand tons during 201415, 1170 thousand tons in 2015-16, and 1276 thousand tons in 2016-17 (Government of Pakistan, 2017).

Chemical agents cause harmful effects on health when meat contains toxic materials, fats, and residues (Mahgoub et al., 2006). The growth rate increases and mortality decreases by the use of antibiotics in the feed of chickens which in return also raises their body weight. It is estimated that 80% of all food-making animals receive medication (Pedreira et al., 2006). Veterinary drugs are still in use at a large scale in the poultry industry with the least control strategies (Hussein & Khalil, 2013). It causes residual deposition in meat tissues and other byproducts (Shahid et al., 2007).

The drug hypersensitivity, allergic reaction (such as anaphylaxis, serum sickness and cutaneous reaction), carcinogenic effect, Hepatic disorders, gastrointestinal and intestinal flora disturbance (Butaye et al., 2001; Hassan et al., 2014), reproductive disorders, human fertility problems, teratogen and mutagenic effect, myelotoxicity, immune-pathological effects, fatal blood dyscrasia (Wadoum et al., 2016) and cancer are those consequences which are caused by different antimicrobial residues in human through meat and meat products (Settepani, 1984; Nisha, 2008; Beyene, 2016;) The food mediums are very complex, therefore highly developed, selective, and sensitive techniques are used for detection. The antibiotic residues are determined by the method of HPLC (high-performance liquid chromatography) (Sadeghi and Jahani, 2013; Aslam et al., 2016). The presence of antibiotic residues in different parts of chicken is against the government policies and regulations of Pakistan (Mumtaz et al., 2000).

The objective and aim of this study were to evaluate the residues of veterinary antibiotics in indigenous and broiler chicken meat. It could be quantified that the residues may because serious health issues to consumers. From this estimation, the research work is done to screen the drug remains in chicken muscle and liver collected from rural areas of

Balochistan. From this perspective, the high-performance liquid chromatography (HPLC) method is selected to detect amoxicillin, oxytetracycline, enrofloxacin, and tylosin respectively.

2 MATERIALS AND METHODS

2.1 Sampling Area

Pakistan has numerous commercial farms of poultry and livestock. Hence, the five different areas of rural Balochistan (Surkhab, Baleli, Hazargangi, Kuchlak, and Bostan) were designated for sampling.

2.2 Sample Collection and Storage

The samples were collected from poultry farms and local houses. Before collecting the samples time of marketing with age was recorded on the first visit, moreover, samples were collected on the same day before the chickens were dispatched to the markets. A total of 160 samples of muscle and liver were prepared from twenty chickens (10 indigenous and 10 broilers) in triplicate. For this purpose, ten indigenous and ten broiler chickens were collected randomly from poultry farms and local houses. The chickens were slaughtered carefully in a butcher shop by the process of conventional neck cut performed by professionals; allow the chicken to bleed for 2 min in a rotary drum picker. Fresh meat was wrapped in plastic sterile and labeled polythene bags then freeze quickly in refrigerators. The samples were brought to the toxicology laboratory of CASVAB (Centre for advanced studies in Vaccinology & Biotechnology), University of Balochistan, Quetta.

2.3 Equipment

HPLC (Sykam, Germany) with UV visible detector (S-3210) and solvent delivery system (S1122) having computer interface was used for chromatography. Other apparatus which was used in the extraction method were Sonicator LC 60 H (Elma, Switzerland), Homogenizer (The Fisher, UK), Centrifuge machine (Hettich, Germany), PES Syringe filters (Aldrich, USA), Centrifuge tubes (NEST, China), Sterile bottles (Nalgene, USA), Vortex mixer (SA3, UK), pH meter (Ohaus, US), Hot plate (VELP, Italy), Digital balance (Panther, USA), Blender (Moulinex, France), Pipette and Micropipette or with disposable tip (Gilson, USA).

2.4 Reagents

All reagents and chemicals which were used in the process of extraction and chromatography were of HPLC grade with at least 99% purity. Citric Acid, nitric acid, AMOX, ENRO, TYL, OTC, 1-propanol, methanol, and SDS solution (sodium dodecyl sulfate) is manufactured by MERCK Germany, formic acid is purchased from the market is

from Across Germany, while Acetonitrile, EDTA (ethylene diamine tetra acetic acid) water-soluble is of the origin of Sigma (Aldrich, USA). Deionized water is prepared in the Deionized section of CASVAB, UOB, and distilled water is prepared in CASVAB through distillation assembly.

2.5 Standard Solutions and Calibration Graphic

The stock solutions of 0.4 µg/ml of Amoxicillin, 0.5 µg/ml of Oxytetracycline, and 0.1 mg/ml of Enrofloxacin were prepared in methanol while 0.1 mg/ml standard of Tylosin was prepared in acetonitrile. These solutions were freshly prepared and stored below 4 °C and the working solution was prepared from stock solutions by diluting them at different concentrations. The parallel dilutions of AMOX were from 0.2 to 25 µg/ml, 0.01 to 50 µg/ml form OTC, and for ENRO and TYL the dilutions were 0.1 to 100 mg/ml with mobile phase just before performing analysis for calibration curves. Each dilution of 20 µl was injected three times in HPLC. The linearity was derived through the correlation coefficient and regression equation shown below in Figure I and Table I.

2.6 Sample Extraction and Chromatographic Conditions

The samples of chicken muscles and livers were cut into small pieces and grinded through a blender. Each sample was weighted in digital balance and put into centrifuge tubes as 4 g for AMOX, 2 g for OTC, 3 g for ENRO, and TYL for extraction. The method was suggested by Ibrahim & Nasr, (2014) for AMOX in which the samples were mixed with 25 ml of (0.05 M) SDS solution by maintaining pH 5 and the solutions were homogenized at 5000 rpm for 10 min. The homogenate solution was sonicated for 15 min and further process centrifuged at 3000 rpm for 10 min. According to Senyuva et al. (2000), 0.1 g citric acid was added in grinded samples, then homogenized for 2 min. 1 ml nitric acid (30%), 4 ml methanol, and 1 ml deionized water were added, then vortex the mixture for 15 min. The suspensions were kept in an ultrasonic bath for 20 min and centrifuged at 4000 rpm for 15 min, respectively for OTC. In the minced samples, 200 µl EDTA (0.1 M) and the mixture of 10 ml methanol and distilled water (70:30) were added. Vortex was used to mix the solution for 30 min, shake manually for 15 min, and centrifuged at 2500 rpm for 10 min. Extract solution of 500 µl was diluted with 2 ml distilled water for ENRO and TYL according to Chico et al. (2008). All the solutions were filtered through a 0.45 µm nylon syringe filter and these filtrates of 2 ml stored in sterile bottles. The composition of the mobile phase consisted of methanol (HPLC grade) and 1-propanol for AMOX, methanol, or acetonitrile and 0.1% of formic acid for OTC, ENRO, and TYL. The aliquots of 20 µl were injected (triplicate) into the HPLC system for analysis by keeping the flow rate at 1 ml/min. The separation was performed on

C18 (5µm × 150mm) with UV detector set at 220 nm, 350 nm, 268 nm, and 282 nm Wavelengths for detection of AMOX, OTC, ENRO, and TYL, respectively. The obtained residues in the samples were quantified and calculated from the area which is under curves interpolated automatically by the Peak-Simple
2.83 software.

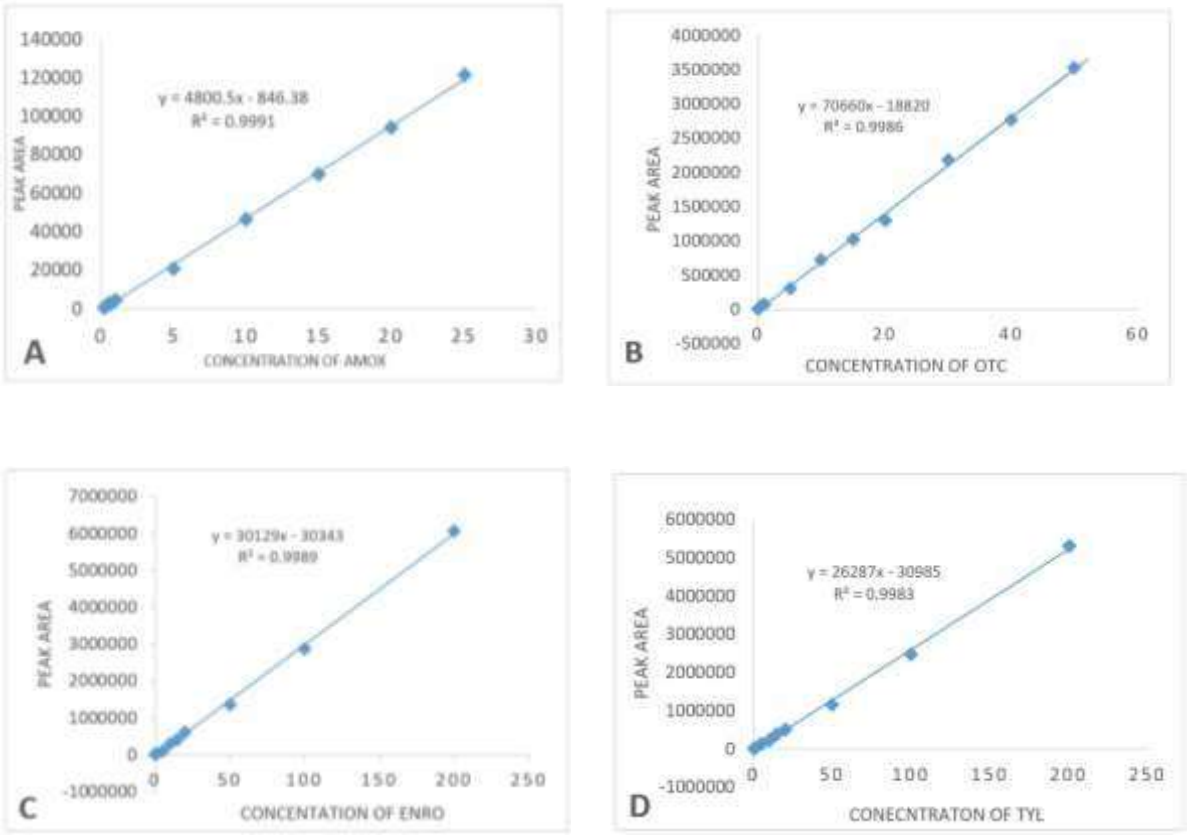

Figure. I: Calibration curve of standard solutions. (A) Amoxicillin 0.2 to 25 µg/ml, (B) Oxytetracycline 0.01 to 50 µg/ml, (c) Enrofloxacin 0.1 to 100 mg/ml and (D) Tylosin 0.1 to 100 mg/ml

Table I: Formula of Standard Calibration and R^2 values of standard solutions.

Antibiotics	Standard calibration Formula: (y = ax - b)		
	a	b	R2
Amoxicillin	4800.5	846.38	0.9991
Oxytetracycline	70660	18820	0.9986
Enrofloxacin	30129	30343	0.9989
Tylosin	26287	30985	0.9983

3. RESULTS AND DISCUSSION

This study represents a random sampling of indigenous and broiler chicken from five different areas of rural Balochistan. To observe the completion of the withdrawal period of antibiotics in commercial chickens is being followed before marketing the flock. Muscle and liver samples were contaminated with antibiotic residues of AMOX, OTC, ENRO, and TYL. Though, the samples in which no residues were detected or were below MRLs were represented as negative samples. The results indicated that out of 160 samples 20% in indigenous and 80% in broiler were having residues of AMOX and OTC in muscles and livers were above MRLs shown in figure II and Table II.

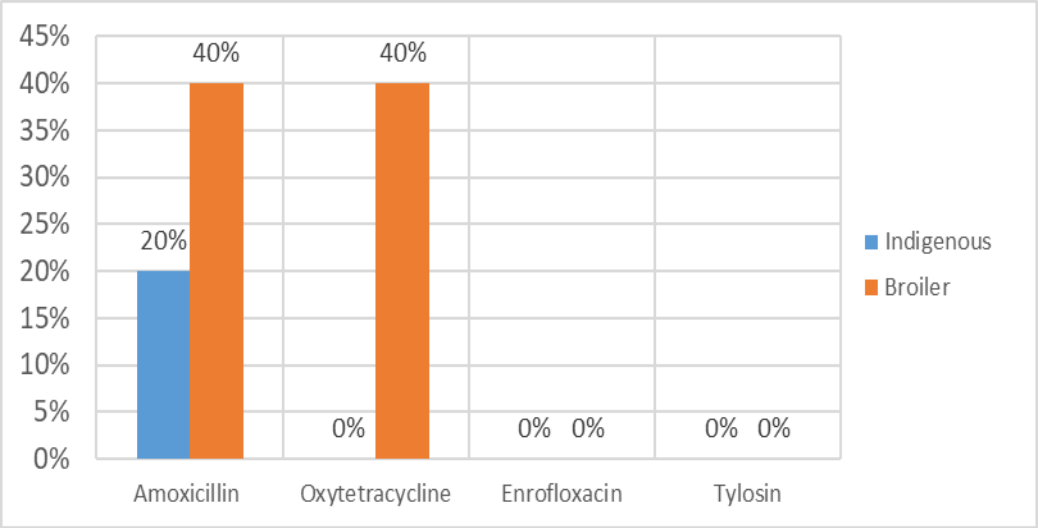

Figure. II: Detected residues of antibiotics in muscles and livers of indigenous and broiler chicken.

Table II: Residual data analyzed by HPLC system in farm and house chickens.

Type of tissue	Total analyzed samples from 20 chickens	Positive samples	Negative samples	Samples below MRL	Sample above MRL
Muscle	80	13	67	3	10
Liver	80	13	67	3	10
Total	160	26	134	6	20

From which residues were not perceived in 134 samples out of 160, whereas from the detected 26 samples only 20 samples were above and 6 samples were below the MRLs. Results shown below in Table III (a, b, c and d) demonstrated that concentration of residues were above the MRLs in muscles ranged from (0.060065 µg/ml to 0.112458 µg/ml) for AMOX and (0.085606 µg/ml to 0.295643 µg/ml) for OTC and in liver ranged from (0.068224 µg/ml to 0.143791 µg/ml) for AMOX and (0.113625 µg/ml to 0.379023 µg/ml) for OTC. Whereas, the concentration of residues of ENRO and TYL was below the MRLs. The concentration is 0.490731 µg/kg in muscle and 0.56201 µg/kg in liver for ENRO and for TYL ranges from 0.413118 µg/kg to 0.774812 µg/kg in muscle and 0.463476 µg/kg to 0.844405 µg/kg in liver. Which also shows the values of LOD and LOQ for AMOX (0.00052 µg/ml and 0.00208 µg/ml in muscles and 0.00085µg/ml and 0.0036 µg/ml in liver), OTC (0.020 µg/ml and 0.0212 µg/ml in muscles and 0.020 µg/ml and 0.025 µg/ml in liver), ENRO (0.426 µg/kg and 1.292 µg/kg in muscles and 0.426 µg/kg and 1.292 µg/kg in liver) and TYL (0.364 µg/kg and 1.104 µg/kg in muscles and 0.364 µg/kg and 1.104 µg/kg in liver), accordingly. The concentration of respective antibiotics is analyzed in poultry farms and desi chickens of five different areas mentioned in table IV.

3.1 Data Analysis

The tentative data were introduced and stored in MS Office word and excel 2016. Results were evaluated for descriptive statistics by using SPSS IBM Software version 22 (Data Editor) released in 2013, IBM Corp, USA.

Table III: Residual level of four respective antibiotics with MRLs, LOD and LOQ

a) Amoxicillin

Sample type	Sample No	Chicken Muscle					Chicken Liver				
		Residues level µg/ml	MRLs µg/ml	LOD µg/ml	LOQ µg/ml	Judgment	Residues level µg/ml	MRLs µg/ml	LOD µg/ml	LOQ µg/ml	Judgment
Indigenous	1	0.065884	0.05	0.00052	0.00208	Rejected	0.078476	0.05	0.00085	0.0036	Rejected
	5	0.060065				Rejected	0.068506				Rejected
Broiler	1	0.081566				Rejected	0.091078				Rejected
	2	0.112458				Rejected	0.143791				Rejected
	5	0.058433				Rejected	0.068224				Rejected
	6	0.088011				Rejected	0.092858				Rejected

b) Oxytetracycline

Sample type	Sample No	Chicken Muscle					Chicken Liver				
		Residues level µg/ml	MRLs µg/ml	LOD µg/ml	LOQ µg/ml	Judgment	Residues level µg/ml	MRLs µg/ml	LOD µg/ml	LOQ µg/ml	Judgment
Indigenous	-	Not detected				Pass	Not detected				Pass
	3	0.216472				Rejected	0.368541				Rejected

Broiler	4	0.085606	0.1	0.020	0.0212	Rejected	0.113625	0.3	0.020	0.025	Rejected
	9	0.295643				Rejected	0.379023				Rejected
	10	0.228982				Rejected	0.351343				Rejected

c) Enrofloxacin

Sample type	Sample No	Chicken Muscle					Chicken Liver				
		Residues level µg/kg	MRLs µg/kg	LOD µg/kg	LOQ µg/kg	Judgment	Residues level µg/kg	MRLs µg/kg	LOD µg/kg	LOQ µg/kg	Judgment
Indigenous	-	Not detected	100	0.426	1.292	-	Not detected	200	0.426	1.292	Pass
Broiler	9	0.490731				Pass	0.56201				Pass

d) Tylosin

Sample type	Sample No	Chicken Muscle					Chicken Liver				
		Residues level µg/kg	MRLs µg/kg	LOD µg/kg	LOQ µg/kg	Judgment	Residues level µg/kg	MRLs µg/kg	LOD µg/kg	LOQ µg/kg	Judgment
Indigenous	-	Not detected	100	0.364	1.104	Pass	Not detected	100	0.364	1.104	Pass
Broiler	7	0.413118				Pass	0.463476				Pass
	8	0.774812				Pass	0.844405				Pass

Table IV: Area of sampling and collected chickens per area.

	Sampling				
Area	Surkhab	Baleli	Hazargangi	Kuchlak	Bostan
Collected chickens	4	4	4	4	4
Broiler	2	2	2	2	2
Indigenous	2	2	2	2	2
Total collected chickens					20

Poultry is one of the main commercial industries of Pakistan which increases 20-25% annually by providing 19% of total meat production and their desolate problem is lack of a proper disease control program (Shah and Korejo, 2012). Besides the developed system of industrial poultry, the rural areas have a substantial position to raise backyard desi chicken (Tufail et al., 2012). This resource also provided food and income for farmers. Commercial poultry has to face numerous diseases and antibiotics are used to treat and prevent diseases and also work as growth promoters stated by Numan et al. (2005) and Shankar et al. (2010). Overuse and misuse of drugs and lack of information about their withdrawal period transfer residual effects to products and consumers. The manifestation of antibiotic residues gains worldwide attention from agencies for public health and various reports specify microbial resistance explained by Yorke & Froc, (2000) and Seri, (2013).

The present study indicated the presence of four different types of antibiotic drugs which were screened by the method of HPLC. For this purpose, samples were collected from five different areas of rural Balochistan from the source of poultry farms and backyard of houses. The comparative analysis shows that indigenous chickens were not treated with drugs although this study depicts that 20% of residues out of 80 had been positively detected and higher than MRLs in indigenous chicken. The reason for the occurring of residues in these samples may depend upon their rearing aura. One of these was nourished in a poultry farm and the other was reared in the house of a dairy farm worker in the same area therefore, this chicken may eat the waste material or feed of dairy animals. On the other hand, the poultry chickens were showed 80% positive samples out of 80 with higher MRLs represented by EU, 2010 which were AMOX and OTC than ENRO and TYL.

The Commission Regulation (EU) was obtained MRLs in food material of animal origin for pharmacologically active substances. The maximum residues limit for AMOX, OTC, ENRO and TYL were 0.05 µg/ml, 0.1 µg/ml, 100 µg/kg and 100 µg/kg in chicken muscles and 0.05 µg/ml, 0.3 µg/ml, 200 µg/kg and 100 µg/kg in chicken liver. The negative samples which were below the MRLs considered as the farmers follow the withdrawal period for the corresponding antibiotic. In this present study the values of LOD and LOQ were followed from Ibrahim & Nasr, (2014), Hussein & Khalil, (2013) and Arslanbas et al. (2018) for AMOX, OTC, ENRO, and TYL, correspondingly.

Emre et al. (2018) evaluated 300 chicken muscle samples, and only 3.6% were found over the allowed MRL and other samples were showed fewer values than MRL for ENRO, DOX, and TYL. Despite this study, Salehzadeh et al. (2007) were examined 270 samples of muscle, liver, and kidney from a slaughterhouse in Tehran, Iran through HPLC system from which 8, 12, and 22 have resulted in higher MRL respectively for residues of ENRO. This result was consistent with the previous study analyzed by Abd El. Monem et al. (2002) and Gad (2012). The report revealed higher values than that attained by Salehzadeh et al. (2006) and Shahid et al. (2007), as their noted residual levels were ranged from 0.030 µg/g to 0.085 µg/g and 0.0066 µg/g to 0.2553 µg/g respectively. Mahmoud and Mohsen (2008) and Shareef et al. (2009) were conquered higher percentages of antibiotic residues like 50% and 56% from the analyzed breast muscles.

In this report liver samples were mostly having higher concentration of residues than the muscles because it metabolises various toxic agents such as 0.060065 µg/ml to 0.112458 µg/ml, 0.085606 µg/ml to 0.295643 µg/ml, 0.490731 µg/kg and 0.413118 µg/kg to 0.774812 µg/kg in muscles and 0.068224 µg/ml to 0.143791 µg/ml, 0.113625 µg/ml to 0.379023

µg/ml, 0.56201 µg/kg and 0.463476 µg/kg to 0.844405 µg/kg in liver for AMOX, OTC, ENRO and TYL respectively. These results supported the description that the liver of chicken accumulated the highest residual concentration of CIP and ENR than muscles reported by Naeem et al. (2006), Islam, (2009), Attari et al. (2014), and Ramatla et al. (2017). Dox and AMOX were observed by Poppelka et al. (2005) and Hussain et al. (2013) in poultry meat by using TLC in the liver, thigh, and breast muscles with several percentages. The most frequently used DOX antibiotic was separated by Jank et al. (2017) in muscles of poultry chicken. Salehzadeh et al. (2006) and Jayalakshmi et al. (2017) were testified OTC and screening the residues in chicken muscle and liver with the highest percentage such as 95.55% and 27.77% respectively.

According to this research, most of the indigenous chicken samples were not detected the antibiotic residues except the samples taken from poultry and near dairy farm shows residues higher than tolerance limits. However, most of the poultry chicken was showed higher residues which consider as positive as allowed limits and others were representing lower values than MRLs which consider as negative observe by the method of HPLC. The positive results which were evaluated are deleterious to human health and the perpetual use of these antibiotic drugs for animal health can be a risk to consumers. This is one of the prevalent issues of the concurrent era which causes horrid health concerns to human beings. There must be an effective and efficient procedure of examination that continuously regulates the application of antibiotic withdrawal period in food origin animals.

4. CONCLUSION AND RECOMMENDATIONS

In conclusion, the results indicate the occurrence of antibiotic residues in poultry meat and products and their contamination in higher concentration than MRL has serious health threats when consumed. This study emphasizes the regulation of use and inspection of antimicrobial medicine vestiges before marketing for monitoring the completion of drug withdrawal time and entirely elimination from the body. Hence, it is indispensable to educate the farmers and local people in Pakistan and inhibit improper administration or unnecessary use of antibiotics and also avoid the use of banned products. As well as there is a key requirement of awareness campaigns among consumers and farmers with the help of veterinarians to minimize the health issues. Use alternative resources for growth promoters such as practice natural antimicrobial agents, encourage probiotics and prebiotics, implementation of good farm management, and provide a hygienic environment for farm and backyard animals. National authorities and public health agencies should also

implement more prudent approaches that animals should be refrained from using the medicines that are being used for human beings to ensure judicious use of drugs in the food of animal origin and prevent the risk of resistance against antibiotics.

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