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Short Communication

Novel Approach for Investigation of Antibiotic Residue in Broilers Grown under Different Agro-Ecological Conditions

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Abstract

The purpose of this research work was to detect antibiotic remnants from chicken meat and to quantify them. For this purpose, the chicken was collected from three poultry farms in Lahore Sheikhupura area. These chickens were categorized according to quality (A, B and C grade) and on the basis of the day of collection (7th, 21st and 35th Growth day). The qualitative analysis was done by agar well diffusion method against *B. subtilis, P. aeruginosa, E. coli* and *S. aureus*. Analysis was done for muscles, kidney and liver samples. The highest residual potential was observed for liver extract (7th day) while the minimum for muscle extract (35th day). The samples with the highest zones of inhibition against pathogens were analyzed by HPLC.

Keywords: antibiotics residue, ciprofloxacin, oxytetracycline, poultry, antibacterial activity

Introduction

Meat is one of the most important constituents of the human diet, as it provides protein, energy, vitamins and minerals [1]. However, meat could also become source of health hazards if it contains excess fats or harmful materials such as toxins or residues of chemical agent [2]. Chicken meat is the most preferred food source in Pakistan. During growth of the broilers, different antibiotics are used which are harmful to human health. There is no direct impression of antibiotic residues in meat on human health, but the risk of generating antibiotic-resistant bacteria may have a potential risk to humans [3-5]. So, it is important to determine the quantity of these residues in chicken meat to set a database for toxic level of these toxins [6, 7].

Antibiotics are all bacteriostatic i.e. small concentration of certain antibiotic can inhibit the growth as well as division of bacterial cells [8]. Antibiotics are employed as a prophylactic agent when

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the animal is known to be more susceptible to infection. Antibiotics are employed as contraceptive measure [9]. The antibiotic left in the foodstuff creates allergic reactions. Antibiotics also causes certain toxic effects. Oxytetracycline & ciprofloxacin are broad spectrum antibiotics, low cost and more easily administered through drinking water or feed [10]. Many bacteria have developed resistance to the available antibiotics due to presence of certain set of enzymes capable to destroy the drug. However, the remnants of the antibiotics causes hazardous effects. The remnants can be detected by some available analytical techniques which varies according to type of residues and type of food to be analyzed [11-14]. Bacterial growth inhibition methods were widely used as screening methods for detecting antibiotic remnants. Their advantage of being relatively in-expensive, rapid and permitted a large number of samples to be analyzed [15-18]. Several tests are available to determine the susceptibility of an organism to a specific antimicrobial drug e.g. agar diffusion test.

The aim of this work is to ensure food safety and protect public health by a reliable screening analysis to determine the level of residual of oxytetracycline and ciprofloxacin as common veterinary antibiotics in chicken meat and chicken luncheon.

Material and Methods

All the chemicals and apparatus employed in this study were of analytical grade. The chicken samples were collected from three poultry farms of Sheikhupura road. The chicken was characterized on the basis of day of collection viz. 7th day, 21st day and 35th day. Moreover, the samples were graded i.e. Grade-A (1-1.5 kg), Grade-B (1.5-2 kg) and Grade-C (2-2.5 kg). The detection of antibiotics was done by checking antibacterial activity against bacterial species using agar well diffusion method. The zones of inhibitions were measured and the samples were further analyzed by HPLC.

For the detection of antibiotics residues of ciprofloxin and oxytetracycline, following method was used to prepare extracts from collected chicken samples. For detection of ciprofloxacin, 5 g chicken sample was ground in a blender to make a fine paste. This paste was homogenized with trichloroacetic acid and centrifuged at 6000 rpm for 15 min. For the detection of Oxytetracycline, the extract was prepared by grinding 5 g chicken meat with mixture of 2 ml citric acid, 2 ml nitric acid, 2 ml methanol and 2 ml deionized water. After mixing it by vortex and ultrasonic bath for 15 min, the sample was centrifuged at 6000 rpm for 15 min. These obtained extracts were used for detection of antibiotics using agar well diffusion method [19]. The medium was prepared by the American Public Health Associations (APHA) standards and ingredients according to Couladis et al. [20].

For the preparation of inoculum strain, nutrient broth was used. Both petri plates and media were autoclaved and media was poured in petri plates in the laminar air flow under aseptic conditions. The bacterial inoculums plates were placed in incubator at 35±2ºC for 24 hours. After incubation, diameter of zone inhibited was measured. Samples of chicken were prepared, diluted to 20 ml and stored at 20ºC. Now 2 ml of different concentrations was added in individual reaction plate, followed by placing a lid on them. These plates were sealed and placed. Afterwards, the suspension of inoculums was streaked on the petri-plates and incubated at 37±2ºC for 24 h. After incubation, petri-plates were analyzed for the presence or absence of microbial proliferation. The HPLC was performed to detect the quantity of the ciprofloxin and oxytetracycline extracts. Distilled water and acetonitrile was used as mobile phase for oxytetracycline group while phosphoric acid and acetonitrile was used for ciprofloxacin group.

The results obtained were analyzed statistically by employing Analysis of Variance (ANOVA) and Duncan's multiple range test using co-stat software (version 3.03; CoHort Software, U.S.A.) to determine the significant value of the analysis, after Steel and Torrei [21].

Results and Discussion

The zone of inhibition demonstrated by the ciprofloxacin and oxytetracycline chicken extracts in opposition to *Bacillus subtilis*, *Pseudomonas aeruginosa, Escherichia coli* and *Staphylococcus aureus* are shown in Table 1. The prepared muscle extract, kidney extract and liver extract of Grade-A, Grade-B and Grade-C chicken samples were analyzed through HPLC which showed the presence of antibiotic residual materials. The peak areas are given in Table 2.

In this study, MRL in poultry meat slaughtered in Lahore-Sheikhupura was evaluated. The average chicken meat consumed each month in the studied region is 2,000 to 2,800 tons. Breast muscles were selected since they do not contain fats and because of their easiness of handling. Furthermore, the widely usage of breast muscle in preparation of different local meat dishes e.g. Shawarma, Thai chicken and Zinger Burger made pectoral muscle tissue a good choice to be screened. Furthermore, kidney and liver samples were also taken into consideration. These antimicrobials are administered to broilers by injections (intramuscularly or subcutaneously) and orally in food or water. Although many antimicrobial groups are used in Sheikhupura, tetracyclines, and fluroquinolenes were chosen to be screened because they are extensively used in poultry medication and can be used in various production periods.

Antibiotics are widely used for growth promoting and nutritive purposes as well as for their therapeutic

Table 1. Representative Inhibition zone produced by chicken samples collected from different poultry farms.

Chicken part	Zone of inhibition against *Bacillus subtilis*					
	Ciprofloxin			Oxytetracycline		
	7th day	21st day	35th day	7th day	21st day	35th day
Muscle	26.66±0.33^{d}	25.66±0.88^{de}	19±0.57^{h}	23.43±0.23^{c}	19.26±0.62^{de}	15±0.27^{f}
Kidney	38.33±0.88^{ab}	21.66±0.33^{f}	19.66±0.33^{g}	35.67±0.78^{b}	20.76±0.13^{d}	16.96±0.53^{e}
liver	58.33±0.88^{a}	50±0.57^{b}	37±0.57^{c}	51.33±0.88^{a}	45±0.77^{ab}	36±0.87^{b}
	Zone of inhibition against *Pseudomonas aeruginosa*					
	7thday	21st day	35th day	7th day	21st day	35th day
Muscle	24.46±0.23^{de}	21.66±0.38^{f}	15±0.57^{h}	29.43±0.23^{c}	25.26±0.62^{de}	19.4±0.27^{f}
Kidney	37.33±0.88^{c}	22.86±0.13^{e}	15.66±0.23^{g}	38.37±0.78^{b}	32.76±0.13^{d}	26.96±0.53^{e}
liver	45.33±0.88^{a}	38.30±0.57^{ab}	32±0.57^{d}	40.33±0.88^{a}	38.45±0.77^{ab}	31.11±0.87^{b}
	Zone of inhibition against *Escherichia coli*					
	7th day	21st day	35th day	7th day	21st day	35th day
Muscle	19.72±0.23^{cd}	15.33±0.35^{e}	11±0.17^{g}	15.34±0.43^{de}	13.23±0.22^{e}	10.11±0.17^{f}
Kidney	21.22±0.88^{ab}	18.32±0.13^{d}	13.22±0.23^{f}	22.54±0.28^{c}	20.23±0.12^{cd}	16.64±0.43^{d}
liver	25.23±0.68^{a}	20.45±0.57^{c}	18.34±0.57^{d}	26.92±0.28^{a}	25.56±0.77^{ab}	23.33±0.87^{c}
	Zone of inhibition against *Staphylococcus aureus*					
	7th day	21st day	35th day	7th day	21st day	35th day
Muscle	18.34±0.33^{cd}	12.44±0.35^{d}	11.34±0.37^{e}	18.32±0.63^{cd}	16.45±0.22^{e}	12.34±0.17^{f}
Kidney	23.45±0.28^{ab}	21.32±0.13^{c}	18.34±0.23^{cd}	20.34±0.28^{c}	18.33±0.12^{cd}	17.45±0.43^{d}
liver	24.45±0.68^{a}	23.33±0.47^{ab}	21.21±0.67^{c}	23.23±0.28^{a}	21.11±0.77^{ab}	20.07±0.87^{c}

Different letters (rows & columns) in superscript are representing different antimicrobial activity for the chicken samples of different categories with respect to their age and body parts.

activities [22, 23] in poultry production. The zone of inhibition demonstrated by the ciprofloxacin chicken extracts of Grade-A in opposition to *Bacillus subtilis* exhibit highest potential provided by the liver extracts of 7th - day chicks sample and least antibacterial capability put forward by muscle extracts of 35th day of chicken growth. *B. subtilis* had offered more resistance against muscle extracts, but found to be susceptible in contradiction to kidney extracts. Liver extracts were found to be most potent against the respective bacterial pathogen. Maximum potential was shown by liver extracts of 7th day sample against *Pseudomonas aeruginosa, Escherichia coli* and *Staphylococcus aureus*.

Quantification of antibiotics of 2 different classes-Oxytetracycline, (class Tetracycline) and Ciprofloxacin (class Fluoroquinolones) were carried out in HPLC. It was observed that all the samples tested were with high amount of antibiotic remnants compared to minimum residual limit (MRL) of American Standards. MRL in poultry meat is as follows: Ciprofloxacin (Muscles: 2.01 µg/ml; Kidney: 2.32 µg/ml; Liver: 2.46 µg/ml) and Oxytetracycline (Muscles: 3.01 µg/ml; Kidney: 3.09 µg/ml; Liver: 3.12 µg/ml). Antibiotic remnants found in tested s amples were shown in Table 2. Each value of the detected peak was calibrated with a standard calibration curve and the residue value was mentioned in µg/ml.

The HPLC results indicates the presence of residual amount of ciprofloxacin, the peak of which appears at retentintion time of 8.20 min compared with standard run under same conditions. Maximum concentration of ciprofloxacin was observed in liver extract. The peak for oxytetracycline appears at retention time of 4.12 minutes. Maximium amount of drug was found in liver extract similar to ciprofloxacin. All the extracts of muscles, liver and kidney showed antibacterial activity against these pathogens that means they had antibiotic remnants in them. The application of antibiotics has led to the fear of the growth of resilient pathogenic bacterial strains. The usage of antibiotic growth promoters was banned in the EU in 2006 due to increasing bacterial resistance and residual risk in animal products (Regulation (EC) No. 1831/2003). The fact that the use of antibiotics as growth promoters has been banned and it is expected that other countries would expand the policy by developing the alternative strategies. Various

Table 2. Antibiotic remnants in microgram per ml of chicken sample.

Antibiotic	Chicken extract sample		Peak area	Antibiotic residue (µg/ml)
Ciprofloxacin	Grade-A	Muscle	67245.33	3.01
		Kidney	94087.11	4.27
		Liver	135841.00	6.23
	Grade-B	Muscle	81305.31	3.67
		Kidney	109851.3	5.01
		Liver	144362.2	6.63
	Grade-C	Muscle	71505.93	3.21
		Kidney	89187.42	4.04
		Liver	130302.2	5.97
Oxytetracycline	Grade-A	Muscle	56421.95	3.33
		Kidney	106334.2	6.57
		Liver	149160.1	9.35
	Grade-B	Muscle	64124.45	3.83
		Kidney	107874.7	6.67
		Liver	147003.4	9.21
	Grade-C	Muscle	68591.9	4.12
		Kidney	122663.5	7.63
		Liver	177813.4	11.21

natural materials have been investigated as effectual replacements to antibiotic growth promoters.

Antimicrobial residues in food of animal origin have received much attention in developed countries to ensure food safety, many countries have monitoring programs to avoid MRL in food of animal origin [24, 25]. Currently, there is no proper system to monitor the presence of MRL in animal products in Pakistan. Therefore, screening of food products from animal origin intended for human consumption for the presence of MRL is essential to ensure food safety [16-18, 26-28].

Conclusions

The study revealed that most of the local broiler chickens contained one or more antibiotic remnants. Antibiotic remnants detected in higher frequency in lighter chickens than heavier ones irrespective of sampling area/farm. It seems that broiler weighing more than two kilograms is the safest category. Withdrawal periods are not observed when broiler chickens are marketed. It was seen that almost all the samples have remnants more than MRL of American standards. This suggested that poultry meat from the studied area was not good for the human health and certain steps must be taken to avoid the remnants of antibiotics which caused serious damage otherwise.

Conflict of Interest

The authors declare no conflict of interest.

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