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Original Article

Determination of Antibiotic Residues in Chicken Tissues Using Four Plate Test Method

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Abstract

Background: Antibiotics are widely used in poultry industry for prevention and therapy purposes. Due to non-compliance withdrawal times and improper use of antibiotics, it is a major public health and food security issue. The administration of antibiotics is one of the primary causes of antimicrobial resistance. It also may cause health problems such as genotoxic, carcinogenic, immunotoxic, or endocrine effects, allergic reactions, and changing natural micro flora of consumers.

Methods: This study focuses on detection of antibiotic residues in the chicken tissue samples. 250 chicken liver, heart, muscle, gizzard, and skin samples were collected from Tabriz slaughterhouse. *Four plate test* as a microbiological method was used for detecting and analyzing antibiotic residues. This method is based on inhibition zone formation around the tissue samples in four culture media with different test bacteria and pH. To investigate the existence of enrofloxacin and ciprofloxacin residues, another extra plate containing Escherichia coli was added to the test. The sample analysis was done by using 1.5 g of each tissue.

Results: Among 250 samples including liver, muscle, gizzard, heart, and skin, 92% of liver samples, 12% of heart samples, 90% of skin samples, and 42% of muscle samples were contaminated by antibiotic residues. All the gizzard samples were free of antibiotics, while the most contaminated tissues were skin samples.

Conclusion: *Four plate test* is an easy and appropriate method for applying in laboratories. The results focus on considering strict legislation for using veterinary drugs in poultry in Iran.

Keywords: Antibiotic residue, Chicken tissues, *Four plate test, Enrofloxacin, Ciprofloxacin*.

1. Introduction

Quinolones and fluoroquinolones are a class of synthetic antibiotics which are used mainly in veterinary medicines. They are responsible for the treatment of variable infections including respiratory, intestinal, urinary diseases, and enteric bacterial infections in animals intended

for human consumption. They have broad spectrum activity against a wide range of Gram-negative and Gram-positive bacteria [1]. Their antibacterial potency is based on a selective inhibition of bacterial DNA gyrase replication and transcription, an essential bacterial enzyme which is responsible for maintenance of spherical twist in DNA [2].

Enrofloxacin(1-cyclopropyl-6-fluoro-1,4-dihidro-4oxo-7-Š4-ethyl-1-piperazinyl-3-quinoline car-boxylic acid) is a synthetic fluoroquinolone antimicrobial agent developed in the late 1980s particularly for using in veterinary medicine. Enrofloxacin is used to treat common poultry infections, such as mycoplasmal infections, colibacillosis, and pasteurellosis [3]**.** The main metabolite of enrofloxacin is ciprofloxacin which appears in different ratios in food after the consumption of enrofloxacin [4]. It is metabolized at a large amount in chicken, but low amounts are detectable [5].

Despite their beneficial effects in treatment of animal diseases, they can cause health problems in animals and human. The presence of antibiotic residues in foodstuff and its transmission to the consumer's body may cause toxic effects. Genotoxic, carcinogenic, immunotoxic, or endocrine effects may be exerted on consumers by intake of these substances [2, 6]**.** The ecology of the intestinal flora of consumers will be changed by the enrofloxacin residue that has entered the food supply. Enrofloxacin is partially metabolized to ciprofloxacin in vivo and change the human intestinal micro flora [7]. Antimicrobial resistance is one of the biggest threats to food security and global health which causes by using antibiotics in farming industries. Allergic reactions are the other important adverse effects of antibiotic residues in food stuff. Penicillin, aminoglycosides, and tetracyclines are the major group of antibiotics which elicit allergic reactions. Skin rashes, allergy, and phototoxic dermatitis have been reported as the result of using tetracycline [8].

The presence of antibiotic residues in animal products including meat and egg has attracted worldwide attention from international and national health agencies**.**

To ensure food safety, the antibiotic residues in foodstuff are determined by applying different methods. Among them, microbial inhibitions tests were the earliest methods used to detect antibiotic residues and they are used widely by now. These methods can cover an extent range of antibiotics. Different assays indicated that these tests have the least false positive results [9].

Different authors report microbial methods for detection of antibiotics. The enrofloxacin residues were determined in chicken tissues in Tehran province, Iran. All samples revealed enrofloxacin residues [10]. In other study, chicken eggs randomly selected from market in Tabriz city and detected antibiotic residues using four plate test. 30% of cases were contaminated by antibiotic residues [11].

The cattle meat was tested by Abdul-Salam Ibrahim *et al*. for monitoring antibiotic residues. 44% of slaughtered meat was positive. The effective surveillance, observance of withdrawal period, control on the use of veterinary drugs are recommended [12].

Ferrini *et al.* used improved microbial test for simultaneous detection of beta-lactams, sulfonamides, tetracyclines, aminoglycosides, macrolides, and quinolones in kidney samples [13].

In the European Union, four plate test is a standard method for detecting antibiotic residues. This method is able to recognize 5 groups of antibiotics including β-Lactam, Tetracyclines, Macrolides, Sulfonamides, and Pencilines.

It comprises three plates of agar medium inoculated with Bacillus subtilis at pH 6, 7.2, and 8 as well as a Micrococcus luteus plate at pH 8. For detecting quinolone families, another plate inoculated with Escherichia coli is needed [14].

The main purpose of this study is to determine the presence of antibiotics especially enrofloxacin and ciprofloxacin in chicken samples in Iran by using Four-plate test method.

2. Material and Method

2.1. Sample collection

In this study, a total of 250 samples including 50 liver, 50 muscle, 50 gizzard, 50 heart, and 50 skin tissues were collected from Tabriz slaughterhouse over six months. The chicken samples were kept at -20 °C for further analysis.

2.2. Chemical materials

Mueller-Hinton agar was purchased from HIMEDIA laboratories are used in a *FPT* method as a culture medium. The pH of the mediums were adjusted at 6, 7.2, and 8 by sodium hydroxide and acetic acid from Merck and controlled by using a digital PH meter (Metrohm, model 733). Then, the cultures were autoclaved at a temperature of 121 °C for 15 minutes. The bacteria used in a *Four - plate test* including: *Bacillus subtilis* (code: PTCC1715), *Micrococcus luteus* (code: PTCC1110), and *Escherichia coli* (code: ATCC10536) were obtained from Iran's Microbial Bank of Pasture Institute. These bacteria were inoculated in nutrient agar culture medium to proliferate, and then their suspensions were prepared in a nutrient broth medium [15].

Bacillus subtilis inoculated at the surface of plates with PH=6, 7.2, and 8. *Micrococcus luteus* and *Escherichia coli* were inoculated at pH=8 and pH=6, respectively.

Antibiogram discs are used as positive control samples and placed at the plates with different pH to ensure the sensitivity of the bacteria using in *Four-plate test*.

2.3. Preparation of samples

The freezing samples of liver, muscle, gizzard, heart, and skin were cut into cylindrical pieces by blade biopsy (No.7), and then discs with 2 millimeter thickness were prepared by scalpel.

Samples were placed on each plate and the name of the bacteria tested, pH of culture medium, the name of the chicken organ, and the number of samples were written on plates. *Bacillus subtilis* and *Escherichia coli* plates incubated at 37 °C for 24 hours. *Micrococcus luteus* plates were placed in oven for 48 hours due to the slow growth of bacteria. Inhibition zones were appeared around positive chicken samples which contained antibiotic residues.

3. Results

In this study, the FPT method is used to detect antibiotic residues especially *enrofloxacin* and *ciprofloxacin* residues in chicken sample tissues including: liver, heart, gizzard, skin, and muscle.

Inhibition zone in four plate test is seen due to remaining of antibiotic residues. Creating inhibition zone at each pH around the samples defines the group of antibiotics which accumulate in the chicken organs. The inhibition zones which were caused by the presence of antibiotic residues in chicken samples are displayed in Figures 1, 2, and 3.

In Four-plate test, 250 chicken samples were studied. The results reveal 46 cases of liver samples (92%), 6 cases of heart samples (12%), 45 cases of skin samples(90%) and 21 cases of muscle samples (42%) have antibiotic residues. As shown in Figure 4 all the gizzard samples were free of antibiotics and skin

samples were the most contaminated tissues. From total 250 samples, 118 samples diagnosed positive, some tissue samples formed inhibition zone in more than one bacteria culture media.

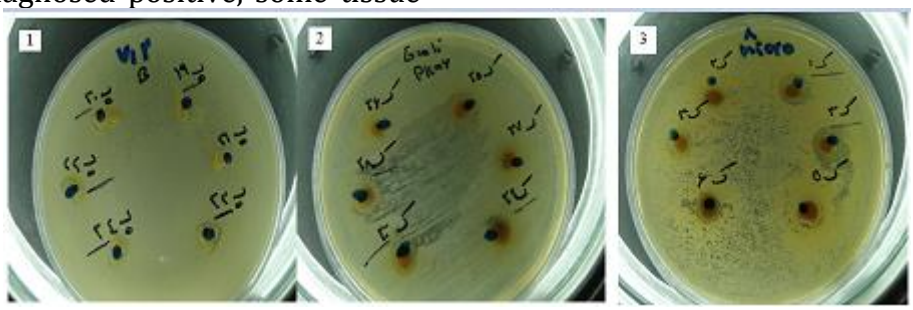

Figure 1. Skin samples (No. 19 to No. 24) with Bacillus subtilis bacteria at pH=7.2
Figure 2. Liver samples (No. 25 to No. 30) with Escherichia coli bacteria at pH=6
Figure 3. Liver samples (No. 1 to No.6) with Micrococcus luteus bacteria at pH=8

On the other hand, 71 cases were inhibited Escherichia coli growth, 63 samples Bacillus subtilis, and 33 cases Micrococcus luteus, respectively. Among all the samples, Enrofloxacin and ciprofloxacin residues were at the highest percentage (72%) in liver samples. Their residues were found in 58% of skin samples and 12% of muscle samples. The number of positive cases of chickens are summarized in Table 1.

Table 1. The number of positive cases of chicken tissues

N	Sample	Number	Number of positive samples				
			Bacillus Subtilis			*Micrococcus Luteus*	*E.coli*
	pH of culture media		pH=6	pH=7.2	pH=8	pH=8	pH=6
1	**Liver**	50	3	0	0	9	36
2	**Skin**	50	18	33	36	15	29
3	**Mussle**	50	6	2	7	5	6
4	**Heart**	50	1	1	1	4	0
5	**Gizzard**	50	0	0	0	0	0

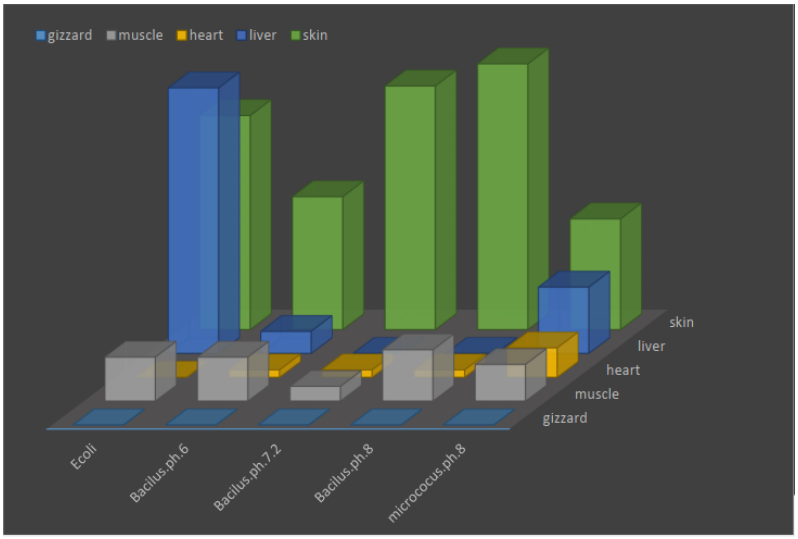

Figure 4. The frequency of antibiotics residues studied in different chicken organs

4. Discussion

The presence of antibiotic residues in food-producing animals has attracted worldwide attention of public health organizations. Due to the use of poultry products as main protein sources, monitoring antibiotic residues in these foodstuffs is an essential issue. Many reports revealed the microbial resistance to antibiotics as a result of animals exposure to these drugs and the resistance may possibly be transmitted to human pathogens [10]. Microbial inhibition assays are the routine methods for screening of foods for residues of antibiotics. These tests are the initial step to show the presence of antibiotics in animal products. These methods are able to detect multiple types of antibiotics. Screening tests should be cheap, simple, easy, and applicable to a large number of samples. These methods should have minimal false positive and false negative results [12, 16]. Four-plate test is a multi-residue screening method which has the potency of detecting antibiotic residues relying on their ability to inhibit growth of sensitive bacteria. The pH of the culture medium as well as of the components of tissue samples, and the type of bacteria affect the obtained results. As indicated in Table 1, the inhibitory zone of antibiotics are depended on pH changes in the medium and also type of utilized bacteria [17].

Using three plates seeded with various bacterial strains (*B. subtilis* PTCC1715, *M. luteus* PTCC1110, and *E. coli* ATCC10536) has an important advantage of detecting multiple groups of antibiotics instead of one group. In some countries, one plate test is used instead of *FPT*. The simple inhibition tests, with a medium and a test bacterium, have a considerable defect as the group(s) of antibiotics involved in the test could not be identified.

Some validated chromatographic methods such as high-performance liquid chromatography, gas chromatography, or hyphenated techniques such as liquid chromatography-mass spectrometry and gas chromatography-mass spectrometry can depict the violation of the MRL regulations, however they have two main problems: (1) These methods need complicated equipment and (2) Each antibiotic family needs its own specific extraction and detection technique. Therefore, the existence of antibiotic residues should be screened with fast and easy microbiological techniques in order to separate contaminated samples, and then test them with chromatographic methods for further analyses [18].

As indicated in Table 1, in liver samples *E.coli* at pH=6, in skin and muscle samples, *Bacillus subtilis* at pH=8 and in heart samples, *Micrococcus Luteus* at pH=8 demonstrated the most frequency of antibiotic residues. Among them, *flouroquinolones* and *macrolides* groups had the most contamination in liver and skin samples, respectively. Detecting flouroquinolones such as *enrofloxacin* and *ciprofloxacin* became possible only by adding one plate of *E.coli* which is not in the classic *FPT* [19]. Enrofloxacin is used extensively in poultry farms, but the recommended withdrawal period, which is prominent for poultry products, is not applied sufficiently [10]. Macrolides are further detected among antibiotic at large amounts. Among them, erythromycin is used commonly to cure Staphylococcus areus arthritis [20].

5. Conclusion

According to the extent needs of protein sources especially poultry products, screening of foodstuff to detect antibiotic residues is a significant matter. These study reveals widespread misuses of antibiotics in chicken tissues and lack of implementation of withdrawal times. The method described here can indicate the residue of enrofloxacin and

ciprofloxacin in chicken samples successfully by adding one plate of Escherichia coli to routine Four-plate test can discover different groups of antibiotics, and also it is an easy and appropriate method for applying in laboratories. The results focus on considering the strict legislation for the use of veterinary drugs in poultry in Iran.

Abbreviation

FPT :Four plate test

Conflict of interest

The authors declare that they have no conflict of interest

Consent for publications

All authors agree to have read the manuscript and authorize the publication of the final version of the manuscript

Availability of data and material

Data are available on request from the authors

Authors contibutions

NR carried out the present survey as the first part of his MSc thesis dissertation in Food Safety and drafted the manuscript. MN and FL supervised the project and participated in review and correction of the manuscript. NA contributed in the correction of the final draft of the manuscript. All authors read and approved the final manuscript.

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Ethics approval and consent to participate

The author did not use any human samples for this study. It does not need an Ethics approval and consent to participate form.

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