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SCREENING OF ANTIBIOTIC RESIDUES IN POULTRY MUSCLE IN LEBANESE RETAIL MARKETS

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ABSTRACT

Antibiotics are used in poultry farms for therapeutic, prophylactic, metaphylactic and nutritive purposes, however the extensive and non-irrational use of antibiotics in livestock production may leave residues that are potentially hazardous to health.

Therefore, this study was carried out on 250 raw meat chicken collected from Lebanese retail market to evaluate the residues level of commonly used antibiotic using three analytical methods: immuno-enzymatic (ELISA) and chemical(HPLC).A questionnaire-based survey identified the different antibiotics used in poultry farms.

ELISA test showed positive results for 65of samples (%), while HPLC detected 78.8 %. Furthermore, the concentrations of antibiotic residues were in the ranges of 6.3-11 µg.$Kg^{-1}$with ELISA, while HPLC showed concentration ranges of 0.01-1.6 mg.$Kg^{-1}$for tetracycline. The Mean of residue levels were above the Codex MRL (or FAO WHO) recommended limit.

These findings confirmed the presence of antibiotic residues in chicken meat samples, Therefore, it is recommended to apply restricted measures and enforcement of regulations to prevent the irrational use of drugs in poultry industry as well as to implement the inspection of chickens for drug residues prior to marketing.

KEYWORDS: Chicken, Antibiotics, Residues, ELISA, HPLC, Maximum residue limit, Lebanon.

1. INTRODUCTION

Chemical and drugs including antibiotics are being widely used in livestock and poultry industries for various purposes such as: improvement of feed conversion efficiency, promotion of animal growth and prevention or treatment of many diseases(Maria Choma, 2003; Dibner and Richards,2005; Salehzadeh*et al.*, 2006; Niewold, 2007; Reig and Toldra, 2008; Hakem, 2013; Hao*et al.*, 2014).

Nowadays, approximately 80% of all livestock receive medication for part, or most, of their lives (De Briyne *et al.*, 2014).

The most commonly used antimicrobials in food-producing animals are β-lactams, tetracyclines, aminoglycosides, lincosamides, macrolides, pleuromutilins and sulfonamides (De Briyne *et al.*, 2014) which can be administered to animal either in feed, in drinking water or by injection (Reig and Toldra, 2008), The extensive use of antibiotics in food producing animals may leave residues in foodstuffs such as meat, milk and eggs(Donoghue and Hairston, 2000; Kan and Petz, 2000).

The major causes for drug residues in food of animal origin might be due to the misuse of antibiotics orfailure to keep the withdrawal period (Nonga*et al.*, 2009; Addo*et al.*, 2010;Salman*et al.*, 2012; Hind *et al.*, 2014).

Human exposure to significant levels of antibiotic residues or their metabolites from animal products may cause numerous health concerns in humans which include allergic reactions, immunopathological diseases (Normanno*et al.*, 2007), carcinogenic effects (e.g., sulphamethazine, oxytetraycyclin and furazolidone), mutagenicity, nephropathy (e.g., gentamicin) bone marrow toxicity, hepatotoxicity, reproductive disorders (Chloramphenicol) (Salehzadeh *et al.*, 2006) and the destruction of useful micro flora present in the gastro-

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intestinal tract (Nisha, 2008; Nonga *et al.*, 2009). Moreover, antibiotic residues may lead to the generation of drug resistant bacteria (Dubois *et al.*, 2001; Tagg, 2013) which has gained its importance due to its ability of transmission to other enteric pathogens and have posed serious public health problems(Tajick and Shohreh, 2006; Nisha,2008; Kim *et al*., 2012; Singh *et al*., 2014; Jechalke *et al.*, 2014).

These adverse effects on human health have led to reject the permission of antibiotic use as growth promoters in the European Union from January 1st, 2006. However, in order to ensure consumer safety, severalorganizations such as the Codex Alimentarius Commission (CAC) has established the Maximum Residue Limits (MRL) for about fifty nine veterinary drugs (CAC, 2014).

Maximum Residue Limit means the maximum concentration of residue resulting from the use of a veterinary drug $mg.kg^{-1}$ or $\mu g.kg^{-1}$ on a fresh weight basis which may be accepted and considered by a community as safe in food animal origin (McGlinchey *et al*., 2008).

It has been proven that the antibiotics used in human medicine either belong to the same general classes or in many cases if they are not exactly the same compounds their mode of action is the same as those used for animals(Gelband *et al*, 2015).

Tetracyclines retain important roles in both human and veterinary medicine. At high level, this antibiotic may cause several adverse public health effects, including allergic reaction, gastrointestinal disorder, tissue damage, and neurological disturbance (Babapour *et al*., 2012).

Tetracycline have been considered for years as an important class of antibiotics in livestock health and production (Kim *et al*., 2013). They have been widely used for the treatment of infectious diseases as well as a growth promoter in animal foodstuffs (Zakeri and Wright, 2008).

The acceptable maximum residues level (MRLs) for tetracyclines as recommended by the Joint Food and Agriculture Organization (FAO)/WHO Expert Committee on Food Additives are 200, 600, and $1200\mu g.kg^{-1}$ for the liver, muscles, and kidney, respectively (Zakeri and Wright, 2008; Babapour *et al*., 2012; Abbasi *et al.*, 2012; Kim *et al*., 2013).

The production of chicken meat in the Middle East was estimated to be 3,415 million tons, 2014 witnessed the peak production (USDA, 2014).

Locally, the production of poultry meat is increasing in Lebanon. The intensive farming system has been developed over the last 10 years.

Lebanon produces around 150 million kilos of broilers (chickens destined for meat consumption) and consumes 30 kg of chicken per capita per year. The country has more than 10 large poultry producers and around 2,000 poultry farms that have the capacity to satisfy the local demand (Daou and Mikhael, 2016).

Currently, Lebanon has no regulations concerning the use of antimicrobials or their maximum residual limits in chicken meat. Therefore, the aim of this study is to determine the presence of antibiotic residues in raw chicken tissue in Lebanese retail market, by using two types of analytical methods commonly used: the screening methods that includes microbiological tests and the confirmatory methods using more advanced techniques such as “enzyme-linked immunosorbent assay (ELISA) and High performance liquid chromatography (HPLC) for specificity and confirmation of results.

2. MATERIAL AND METHODS

Questionnaire-based survey on Lebanese poultry farms

A questionnaire-based survey was carried out between January and March 2016 on one hundred poultry farms covering most Lebanese region and was targeted to either owners or managers of the farms. The questionnaire consisted of a brief introduction, which clarifiedthe objectives and the importance of respondents’ participation.The rest covered questionsrelated to1) Size and type of poultry farms2) diseases currently on the

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farm3) Identifying the most commonly used antibiotics4) frequency and reasons of use, 5) withdrawal period 6)vigilance of poultry farmers on the use of antibiotics and related problems, and other appropriate information on antibioticusage.

Ethical considerations
The questionnaire was approved bythe ethical Committee of the Holy Spirit University of Kaslik.

Materials
Sample Collection
A total of 250 samples of Chicken muscle were collected from various Lebaneseretail markets during the period extended from March 2016 to January 2017. Samples were stored in sterile bags at -22°C for further analysis.

Evaluation of antibiotic residues
Two methods were used for the detection of antibiotics residues in raw chicken meat at the laboratory of the faculty of Agricultural and Food Sciences at the Holy Spirit University of Kaslik.

- Quantitative Method:
    a) Enzyme-Linked Immunosorbent Assay (ELISA)

The ELISA analysis of the antibiotic residues of the tetracycline was carried out according to the manufacturer's guidelines(ELISA kit; r-biopharm, Darmstadt, Germany). The kit used was a competitive enzyme immunoassayfor the quantitative analysis.
The specificity of the tetracycline kit was done by analyzing the appropriate substances in buffer system. The limits of detection (LOD) and recovery rate of this test in meat were approximately 2 ppb and 99% respectively.

    b) High performance liquid Chromatography (HPLC)

The optimization of detection and quantification of tetracycline residues were done using UV-HPLC according to Thermo UHPLC method. Gradient elution was completed using a stationary phase C18 (150 x 4.6 mm) column. The mobile phase is a mix of 50%Ammonium dihydrogen phosphate20mM + 50% acetonitrile, was flowed at 1 ml/min rate. The wavelength was adjusted at270 nm. The injected volume was 10 µl and the run time was about10 min.

Statistical Analyses:
IBM SPSS (version 23) statistical software was used for statistical analysis. Chi -square test and T-test were used to determine the significant differences between the antimicrobial use and residues at $p < 0.05$.

3. RESULTS

Questionnaire results
One hundred farms have completed the survey. The questions and the results of the questionnaire, were presented in Table1.

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Table 1: Survey res

Group variable	Unique value	Number of farm
Location	Bekaa North South Mount Lebanon- Chouf Jbail	13 52 6 9 4 6
Antibiotics use	yes No	100 0
Choice of antibiotics	Veterinary prescription Drug dealer prescription Availability Personal selection (Efficacy)	85 4 5 6
End of administration	Disappearance of symptoms End of the recommended amount of the drug	27 73
Dosage	Estimation Weighing with scale Veterinary instructions Following sheet	9 54 22 15
Withdrawalperiod	Yes No	0 100
Purpose	Therapeutic and prophylactic Therapeutic	66 34
Type of antibiotics	AmpicilineAmoxicillinCefalexinChloramphenicolCiprofloxacinColistinDoxycyclin Doxycyclin+gentamycin=dox/gEnrofloxacinErythromycin fosfomycinFuraltadonNeomycinNorfloxacin OxytetracyclinStreptomycinSulfadiazinTetracyclinTilmycocinTrimethoprimTylosin	2.9 % 12.1% 5.9 % 0.7 % 1.7% 8.1 % 9.5 % 0.7 % 6.2% 2.3% 5.9% 3.9% 10.6 % 6.1% 4.5% 1.4 % 0.5% 0.7 % 1.4% 0.5% 13.4%

Broiler farms in 6 different locations were surveyed. 52% of farms were in North Locality and the remainder (48%) was distributed in all Lebanon.

The majority of the interviewed farmers were large-scale producers who had a range of >2000 birds (95%). Chickens were slaughtered at an age of 40 days. Concerning the use of antibiotics, most of the farms (66%) use antibiotics for treatments and for prophylactic purposes.

These results also indicate that the family of tetracycline's(15.4%), Tylosin (13.4%),Amoxicillin (12.1%), Neomycin (10.6%) and Colistin (8.1 %), were used commonly in farms.

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All farmers indicated that antibiotics are administered in feed and water. Mainly, the treatment is stopped at the end of the recommended amount of the drug (73%).The association (percentage) between what famers reported, and the detection of antimicrobial residues (tetracycline in our study) was studied.

The majority of farmers indicated that 7 days is enough to be considered as withdrawal period. None of the answers were shown to be statistically significant.

Detection of Antibiotic Residues in Meat Samples Using ELISA Methods

The results of the ELISA test to determine the presence and concentration of Tetracycline in the sampled broilers are exposed in table2.

Table 2: Distribution of tetracycline residues using ELISA method

Region	Effective	AVERAGE ($\mu g.Kg^{-1}$) ± SE (Standard Error)
Baabda	38	9.30 ± 1.30 ^{ab}
Beirut	22	6.30 ± 0.60 ^{a}
South	11	9.20 ± 0.70 ^{ab}
Bekaa	41	8.20 ± 0.50 ^{ab}
North	50	8.80 ± 1.20 ^{ab}
Kesrouan	56	10.20 ± 1.20 ^{ab}
Metn	32	11.00 ± 1.20 ^{b}
Average	250	9.00 ± 0.90

On the overall, ELISA analysis showed that 65% of the samples tested positive for tetracycline residues. The tetracycline levels in the samples are ranged from 6.30 to 11.00$\mu g.Kg^{-1}$ with overall mean concentration is9.00$\mu g.Kg^{-1}$ with a significant difference ($p < 0.05$) between regions.

This test verifies and confirms results previously obtained from chicken tissues where the concentration of tetracycline is relatively high.

The lowest contamination is in chicken collected from Beirut area (6.30 $\mu g.kg^{-1}$);while the highest contamination is observed in chickens from Metn region with a concentration of 11.00 $\mu g.kg^{-1}$. There is no significant differences of results between regions.

Quantitative Analysis of Antibiotic Residues in Meat Samples Using HPLC Methods

The positive samples from ELISA and microbiological inhibition assay methods were further analyzed using HPLC for quantification of tetracycline residues. 197samples from the 250 analyzed samples (78.8/%) revealed a detectable level for tetracycline residues. Theresidual concentration ranged from 0.01$mg.kg^{-1}$ to 1.6 $mg.kg^{-1}$asshown in table3, with a mean value of 0.123± 0.091 $mg.kg^{-1}$.

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Table 3: Distribution of tetracycline residues using HPLC method

Region	samples collected 250	Positive samples%	Tetracycline Concentration								samples>LMR* %
			0.01-0.03 mg.kg^{-1}	0.04-0.06 mg.kg^{-1}	0.07-0.09 mg.kg^{-1}	0.1-0.3 mg.kg^{-1}	0.4-0.6 mg.kg^{-1}	0.7-0.9 mg.kg^{-1}	1.1-1.3 mg.kg^{-1}	1.4-1.6 mg.kg^{-1}	
			Range (%)								
Baabda	38	65.8	13.2	10.5	0.0	28.9	5.3	2.6	2.6	2.6	42.1
Beirut	22	100.0	50.0	13.6	9.1	22.7	4.5	0.0	0.0	0.0	27.3
South	11	63.6	0.0	18.2	9.1	18.2	0.0	0.0	0.0	18.2	36.4
Bekaa	41	82.9	22.0	22.0	9.8	12.2	7.3	4.9	2.4	2.4	29.3
North	50	70.0	10.0	22.0	18.0	6.0	4.0	0.0	2.0	8.0	20.0
kesrouan	56	82.1	25.0	5.4	8.9	30.4	3.6	5.4	3.6	0.0	42.9
Metn	32	87.5	21.9	18.8	9.4	31.3	3.1	3.1	0.0	0.0	37.5

The chromatographs of the reference standard and calibration curve for tetracyclinesare shown in Figure 1. The calibration curve for tetracycline showed good linearity, r^2 = 0.999581. The limit of detection(LOD) is 0.01mg.kg^{-1}for the antibiotic residues analyzed.

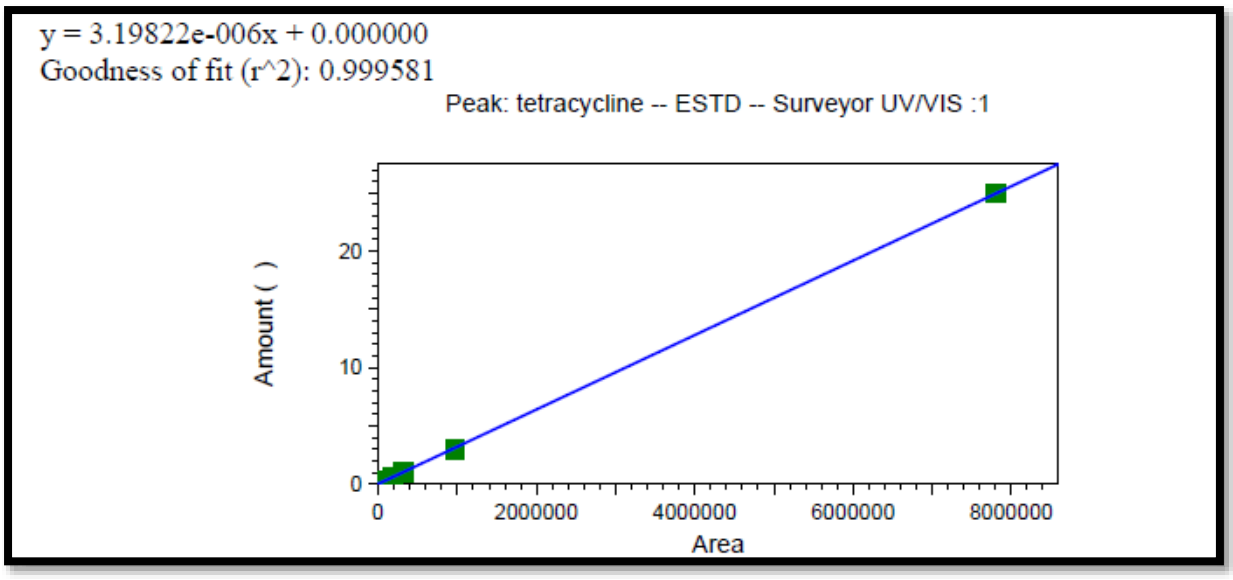

Figure 1: standard curve of tetracycline standard.

The highest percentage of antibiotic residues was detected in Beirut (100%). The lowest percentage of residues were detected in Baabda(65.8%). The percentage of samples that have been above the LMR is 33.2 % (Table3).

4. DISCUSSION

Antimicrobial residues in food of animal origin have received much concern in developed countries to guarantee food safety (Ellis, 2008).

Currently, Lebanon has no clear researches regarding the use and control of antimicrobial drugs in food. Therefore, screening of antibiotic residues widely used in poultry production is necessary to avoid potential toxic effects on human and animal health.

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Questionnaire

It is obvious that most of farms continually used antibiotics as a prophylaxis and with greater frequency during disease outbreaks. It is well noted that antibiotics are administered during the breeding of broilers, either in the feed as a growth promoter or orally in drinking water for treatment of infections.

In this study, it was found that antibiotics, in particular tetracyclines family and tylosinare used extensively in poultry production. Tylosin is a macrolide antibiotic approved for poultry as a drinking water medication due to itslarge spectrum of activity against Gram positive bacteria including *Streptococci*and *Staphylococci*, but a narrow spectrum against Gram negative bacteria such as *Campylobacter*and against *Mycoplasma gallisepticum*, the relevant agent of Chronic Respiratory Disease in poultry (Annan-Prah *et al.*, 2012).

Although tylosin is added to feed to stimulate gain weight and improve feedefficiency, it is not permitted for use as a feed medication for poultry in Canada and European countries (Phillips, 1999; BAM, 2014).

Tetracyclines are broad-spectrum antibiotics used to treat a variety of infections in human medicine and are also used as growth promoters in animals. About 60% of an ingested dose of oxytetracycline is widely distributed from the gastrointestinal tract to the whole body, particularly to liver, kidney, bones, and teeth (Doyle, 2006).

Although few numbers of farmers bought drugs on prescriptions, it was clear that most of farmers, made their own diagnosis of diseases that were occurring and decide which antibiotics to use by themselves.

On the other hand, our findings recorded that some of the antibiotics that were given neither provides information about their active components nor their withdrawal periods. This generally happened with not approved antibiotic products which could access the country by unofficial path to escape Customs authorities. (Annan-Prah *et al.*, 2012).

Without forgetting that veterinary drug suppliers do not recommend regularly certified veterinary prescriptions before making sales.

Additionally, the density of broilers in farms plays an important role in breeding and could increase proportionally the dose of antibiotics added to the diet in order to reduce the stress of the environment.

It is important to notice as well, that after each production of a broiler batch (35-45 days of breeding) allLebanese farms apply total cleaning and disinfection during period of 10 to 15 days. N'kaya (2004) in his research specifies that the time of rest and disinfection must be greater than 15 days, in order to eliminateall microorganisms in livestock buildings, otherwise contamination by pathogens is more likely and requires the application of high doses antibiotics.

All these factors, result in an irrational use of antibiotics, inadequate combinations and fast substitution to other drugs and inappropriate dosage(Annan-Prah *et al.*, 2012). Whichit is important to control the concentration of these residues in order to guarantee that they do not surpass the MRL and to avoid the emergence of antibiotic resistant strains of bacteria and cross resistance with other bacteria (World Health Organization, 2014).

Farm managers should implement biosecurity measures and good antibiotic management through applying the appropriate administration, storage and withdrawal periods upon veterinary consultancy and prescriptions.

Determination of tetracycline by ELISAmethod (quantitative method)

This study confirms the ability of the ELISA method for analyzing the tetracycline residues in chicken meat with high sensitivity. Values were above the MRL, the differences in concentration levels of tetracycline noted in this study between the ELISA and HPLC methods could be due to the inability of the ELISA method to separate between the tetracycline groups.

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Determination of tetracycline by HPLC Analysis

In order to confirm and determine the occurrence of tetracycline residues in chicken meat, the HPLC method was used after preliminary qualitative microbiological screening and quantitative analysis using ELISA method.

The concentrations of tetracyclines in many samples (33.2% above MRL) exceeded the maximum limitestablished by regulatory authorities as European Union (2010) which is 100 µg.Kg^{-1}. The obtained results were comparable to those reported in a study in Taiwan (Su-Ching *et al.*,

2016) showing that more than 49% of farmers sell their products within withdrawal periods. A study by Al Ghamdi (2000) in Saudi Arabia shows that residues were detected in 47.6% of the analyzed muscles, whose 82% contain concentrations higher than the MRL.

Moreover, results reported in our study were consistent with these previously found by Gad (2012), but higher than that obtained by Salehzadeh *et al.*(2006) and Shahid *et al.* (2007), who reported a residue level ranging from 0.0066 to 0.2553 and from0.030 to 0.085 µg.g^{-1} respectively.

The detection of tetracyclines in oursamples, reflect their frequent use in veterinary prescriptions and in intensive poultry production. According to Corpet and Brugere(1995) and Bonfoh *et al.* (2003)and Ouattara *et al.* (2013)the presence of these antibacterial residues could be due to the treatment of poultry followed by an insufficient withdrawal period(Okerman *et al.*, 2007; Mahmoud and Mohsen, 2008).Tetracyclines have a withdrawal period of 21 days, which makes it difficult for farmers to wait this period, this issue may lead toantibiotic residues in animal food origin exceeding the MRL.

In an investigation of the presence of antibiotic residues in meat sold in Dakar(Donkor *et al.*, 2011) detected macrolides and/or beta-lactams in 77% of samples, and aminoglycosides in 7.87%. These results showa diversity of molecules used in poultry farms.

Antibiotics are essential substances in veterinarymedicine; however, a*long exposure to* these moleculesis apotential source of toxicological, allergic(Nisha, 2008) and antibacterial resistance risks (Espinasse, 1993; Sanders, 1999). Besidesthese health problems, the incidence of drug residuesin food of animal origin is an important economic barrier in international tradewith the new sanitary and phytosanitary rules ofthe World Trade Organization(Maron *et al*, 2013).

Some researchers found that antibiotics as growth promoters have either none orminimal benefits (Emborg *et al.*, 2001; Engster *et al.*,2002; World Health Organization, 2014). Furthermore, USDA (2009) assumed that animal production could be enhanced by improved hygiene in animal houses and improved disease surveillance.

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