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Studies on Residues of Antibiotics and Growth Enhancer-Hormone in Imported and Locally Produced Beef

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Abstract

A total of sixty random samples of fresh local and frozen imported beef (30 of each) were collected from different places in Al-Menoufia governorate, Egypt for estimation of their antibiotics residues (oxytetracycline "OTC" and penicillin) by using high performance liquid chromatography (HPLC) and enzyme-linked immune sorbent assay (ELISA) for hormonal residues. In the present study, antibiotic residues either oxtetracycline or penicillin in beef were recorded at higher concentrations in the examined samples of locally produced beef than the imported beef, where 5 (16.67%) and 3 (10.00%) samples were unaccepted as their residues content exceeded the (MRL) recorded by Egyptian Organization for Standardization and Quality; 200 µg/kg and 50 µg/kg for oxtetracycline and penicillin respectively. In contrast, the highest concentrations of testosterone hormone residues were recorded in the imported beef samples and its level in 5 (16.67%) samples exceeded the MRL recommended by FAO/WHO. Application of various cooking methods (boiling, grilling and microwave) on beef of each category (n = 5) exhibited that cooking methods positively reduced residues of OTC, penicillin and testosterone. The obtained results revealed that the most effective cooking methods for reducing the levels or elimination of such antibiotics and hormone in beef were grilling followed by microwave and then boiling.

Keywords: Antibiotic residues; Growth enhancer-hormone; Oxytetracycline; Pencillin

Introduction

Antimicrobials are administered to animals either by injections, orally in feed or water, topically on the skin, intra mammary or intrauterine infusions to control, prevent and treat infection as well as to enhance animal growth and feed efficiency. Theoretically, all of these routes may lead to residues appearing in foods of animal origin such as milk, meat and eggs [1]. Oxytetracyclinesare the most predominantly prescribed antibiotics in Africa, and they represent 41% of cases, followed by β-lactams at 18% [2]. Antibiotic residues in food have been linked to growing public health concerns over the spread of antibiotic resistant microorganisms, human allergic reactions and imbalances in intestinal microflora. Moreover, their presence may affect fermentation processes in food production industries [3]. The use of anabolic agents is a common practice adopted in livestock in several countries to increase meat production by stimulating the protein synthesis and improving the feed conversion. In those countries, the most common compounds used are the natural anabolic agents (testosterone) and the synthetic anabolic agents (trenbolone acetate). In Brazil, these compounds are prohibited due to regulations setly the external markets and the possible risks to the public health [4,5], stipulated that in light of the carcinogenic potential and their residues and obvious human health risks, the European Community forbade the use of steroids as growth-promoting agents in livestock breeding. There are many common methods used to cook meat; the most popular are boiling, microwaving or grilling. However, grill was found to be the sole cooking method that reduced sex steroid hormone residues in meat with a level of reduction approached 31% of that found in raw non cooked meats [6]. Due to the excessive use of antibiotics in raising fattening animals for human consumption and the implementation of growth promoters in some animal farms, our study focus on the monitor of residues of such chemical compounds to figure out if these residues fall in the accepted MRLs and measuring the effect of various popular cooking method in reducing or elimination of such residues in meat. Moreover the observed findings may be helpful in confirming and selecting the ideal method for cooking so as to effectively reduce antibiotic and hormone residues in meat prior to consumption

Materials and Methods

Part 1: Determination of antibiotics and hormone residues

Sixty random muscle samples of fresh local and frozen imported Brazilian beef of the same cuts (Thigh muscles); 30 of each were purchased from different supermarkets at various localities in Menofia governorate, Egypt. The samples were collected at different periods of time within their validity date. Each collected sample was separately placed in clean sterile polyethylene bag and transferred in an ice box as quickly as possible to the laboratory for estimation of some of their antibiotics and hormonal residues.

Determination of antibiotics residues in beef samples

The detection of oxytetracycline and penicillin residues was applied by using the technique of High Performance Liquid Chromatography (HPLC).

Determination of oxytetracycline [7]

Extraction of the drug from the sample: All samples were finely cut with scissors after trimming of the external fat and fascia. Two grams of each examined meat sample to be analyzed was cut into very small pieces and subsequently ground into fine particles using Sartorius mincer. This

was then homogenized in a blender for 2 min. before 0.1 g of citric acid was added. One ml of nitric acid (30%), 4 ml methanol and 1 ml deionized water were added to this mixture, respectively. The suspension with solid particles was put in a vortex for good mixing, kept in an ultrasonic bath for 15 min and centrifuged for 10 min at 5300 rpm. After filtering through a 0.45 µ nylon filter, 20 µl of clear solution was injected into HPLC for analysis.

Chromatographic condition: The mobile phase of methanol and formic acid (0.1%) using a gradient method with a flow rate of 1.5 ml/min at 25°C was applied. The separation was done on Hypersil gold C18 (10 µm, 100 × 4.6 mm) columns with mobile phase as described above. Detection was performed with PDA detector set at 350 nm wave length. Quantification of residues in samples was obtained and calculated from areas under curves extrapolated automatically by the software (Chromo Quest 5).

Calibration curve: The curve was prepared by using concentrations of 10, 20, 30, 40, 50 and 60 µg/L of oxytetracycline in eluent. These standards were prepared from the daily prepared stock solution and treated as 100 mg of oxytetracycline standard was accurately weighed and put in a 100 ml volumetric flask. The powder was dissolved in 100 ml of methanol to make a stock solution. The detection limit for oxytetracycline was 0.01 ppm, while the retention time was 3.9 minutes. The concentrations of antibiotic residues in the samples were calculated with reference to a calibration curves obtained from work solutions of oxytetracycline. For the preparation of the work solutions, Oxytetracycline hydrochloride (Sigma Aldrich, Inc., St. Louis, MO, USA) stock solutions (1 mg/ml in methanol) of the antibiotics were diluted to several concentrations by using methanol as a diluent.

Determination of penicillin residues [8]

Extraction of the drug from the sample: Accurately, 5 g of the sample were put into 50 ml capped polypropylene centrifuge tube and to which 15 ml of acetonitrile/water (15:2) mixture was added. Complete homogenization of the sample for one minute with centrifugation at 4000 rpm. The supernatant was taken for repeating the homogenization and centrifugation one additional time. Furthermore, the combined supernatants were placed into a round bottom flask and the acetonitrile was evaporated at 37°C. At least, approximately 6 ml of remaining supernatant should be found in the flask. The total volume of supernatant should reach 20 ml using phosphate buffer (pH 8.5). The supernatant was filtered through regenerated cellulose, 25 mm, 45 urn syringe filter (Agilent pin 5185-5831). Actually, 10 ml of the extract were loaded onto the Agilent Sampli Q OPT 6 mL/150 mg cartridge

Purification [9]: The cartridge was washed with 0.1% formic acid in water and then with (*p*H 8.5) potassium phosphate buffer. Finally, the sample was eluted with 3 ml acetonitrile. The sample was filtered with a 13 mm, 45 ml poly tetra fluoro ethylene (PTFE) syringe filter (Agilent pin 518-5836). The eluent was dried under nitrogen at room temperature. The residue was resuspended in mobile phase to 1.0 ml. The sample was vortexed for 2 minutes and then transferred to a 2 ml, auto sampler vial (Agilent pin 5182-0864).

Solid-phase extraction (SPE) and final sample preparation: The SPE cartridge was placed into vacuum manifold system with SPE cartridge effluent going to a solvent trap. Accurately, 25 ml of methanol, 25 ml of water and then 40 ml 0.01 M calcium hydroxide were added. Flow rate is not important for these steps. Furthermore, 3 ml of the sample was applied to the cartridge with a flow rate not more than 2 drops. The cartridge was not allowed to dry at this step. The cartridge was flushed with 40 ml distilled water and then 10 ml acetonitrile. Elution was performed successively with 40 ml of 2.5% acetic acid including 50% methanol. The collected elute was evaporated using rotator evaporator at 45°C till complete dryness. The dried residue was reconstituted in 3 ml of the mobile phase. The samples were mixed and filtered through 0.2 µm filters before injection into the LC system.

Separation of penicillin on the solid phase by HPLC: High performance liquid chromatography (HPLC) used for antibiotic determination was an Agilent 1100 HPLC system at Animal Health Research Institute in Dokki, Agilen Technologies, Waldbronn, Germany, equipped with quaternary pump model G 1311A, UV detector (Model G 1314A) set at 254 nm wavelength, auto sampler (model G1329A VP-ODS) and Shim pack (150 × 4.6 mm) column (Shimadzu, Kyoto, Japan).

Determination of testosterone residues by ELISA [10] **Reagents and materials supplied in kits: (EIA Methyl testosterone 2 hours manual kits)**

Preparation of samples: To 1 g of homogenized beef sample, 1 ml of 67 mM phosphate buffer (pH 7.2) was added and vigorously shaked for 5 minutes.

Extraction of samples: Accurately, 5 ml of Tert-Butyl-Methyl Ether (TRME) was extracted and after shaking for 30-60 minutes and centrifugation for 10 minutes at 3000 rpm. The supernatant was transferred to another tube and the extraction was repeated with 5 ml Tert-butyl-methylether. The ether phase was pooled and reduced to dryness at 40°C. The residue was dissolved in 1 ml methanol/ distilled water (80/20).The sample was evaporated until dryness at 60°C. Further, the residue was dissolved in 200 µl of ethanol and 1.8 ml of dilution buffer. (Methyl testosterone concentration in the sample=methyl testosterone concentration in the extract x 4)

Preparation of reagents

The technique recommended with the applied kits was carried out. The standard and blank solutions were provided in concentrations of 0, 0.05, 0.1, 0.2, 0.5, 1 and 2 ng /ml to construct the standard curve.

Part 2: Experimental Part (Heat treatment)

The main purpose of the present work is to investigate the effects of certain common cooking methods on the concentrations of both antibiotic and hormonal residues in question. Accordingly, 5 positive samples of beef containing low, medium (around permissible limit) and high concentrations of oxytetracycline, penicillin and testosterone were subjected to the various cooking methods used at home. Sample weighing 10 gm, with thickness 2.5 cm and core temperature of 71.5°C were used. Accordingly, treatment of boiling at 100°C for 30 minutes, grilling at 200°C for 15 minutes and microwave at 180°C for 10minutes) were applied on the positive samples which proved to contain oxytetracycline, penicillin and testosterone residues to determine the efficacy of each cooking method on the stability of the such serious residues.

Results and Discussion

The obtained results were stastically evaluated by application of student t-test according to Feldmanet et al. [11].

The results given in Table (1) and Figure (1) revealed that oxytetracycline (OTC) residues were detected in 10% and 36.67% from examined imported and local beef samples, respectively. Moreover, the levels of OTC residues (µg/kg) ranged from 12.7 to 56.0 with a mean value of 35.1 ± 2.24 for imported beef and 17.5 to 601.8 µ with a mean value of 212.5 ± 14.96 for local beef. The difference between the examined samples of imported and local beef were highly significant (P<0.01).According to the Egyptian Organization of Standardization and Quality "EOSQ" No. 3692, the MRL of OTC in meat is 200 µg/kg. (Table 1) and (Figure 2) declared that the imported beef samples were accepted where their OTC residues was

within the permissible limit, in contrast, 16.67 % of the examined local beef samples were unaccepted .The present results agree, to some extent, with those recorded by Olatoye and Ehinmowo and Mehran et al. [12,13] who recorded that the mean value of the total TCs residues in triceps muscle samples was 176.3 ± 46.8 µg/kg. Higher results were obtained by Muriuki et al. [14] who recorded that the mean TCs levels of samples in Athi River, Kenya were 280 µg/kg. However, lower results were reported by Adesokan et al. [15] who recorded that the mean amount of OTC residues in muscle was 16.17 ± 5.52 µg /kg. Tetracyclines have a broad range of activity against variety of Gram positive and Gram negative bacteria and of low price, for these reasons, TCs are widely used in veterinary medicine for preventing and treating several diseases and for promoting growth in cattle [16]. The residues of OTC may pose a health threat to consumers, depending on the type of food and amount of residues. The human health problems resulting from intake of sub chronic exposure level of OTC include gastrointestinal disturbances [17]. OTC residues possessed a teratogenic risk to the fetus with allergic reaction [18] and help in development of resistant pathogens for human and animals [19]

Results achieved in (Table 2) and (Figure 1) declared that penicillin residues were detected in 26.67% and 23.33% of the examined samples of imported and local beef, respectively. Moreover, the levels of penicillin residues (µg/kg) ranged from 3.6 to 24.1 with a mean value 12.10 ± 0.93 for imported beef and from 5.9 to 71.3 with a mean value 36.57 ± 4.08 for local beef. The differences between the examined samples of imported and local beef were highly significant (P<0.01).According to the "EOSQ" No. 3692 (2008) [20], which recommended that the MRL of penicillin residues in meat is 50 µg /kg, so Table (2) and Figure (2) revealed that the examined samples of imported beef were accepted where their penicillin residue was

		Imported beef	Local beef
Positive samples	**No.**	3	11
	%	10	36.67
Minimum		12.7	17.5
Maximum		56	601.8
Mean ± S.E		35.1 ± 2.24	212.5 ± 14.96++
MRL/(µg/kg)		200	200
Accepted samples	**No.**	30	25
	%	100	83.33
Unaccepted samples	**No.**	0	5
	%	0	16.7

Table 1: Statistical analytical results and acceptability of the examined samples of local and imported beef according to their contents of oxytetracycline residues (µg/kg) (n = 30).
++ = High significant differences (P< 0.01) as indicated by t-test.

		Imported Beef	Local beef
Positive samples	**No.**	8	7
	%	26.67	23.33
Minimum		3.6	5.9
Maximum		24.1	71.3
Mean ±S.E*		12.10 ± 0.93	36.57 ± 4.08++
Maximum Residual Limit (µg/kg)*		50	50
Accepted samples	**No.**	30	27
	%	100	90
Unaccepted samples	**No.**	0	3
	%	0	10

Table 2: Statistical analytical results and acceptability of the examined samples of local and imported beef according to their contents of penicillin residues (µg/kg) (n = 30).
++= High significant differences (P<0.01) as indicated by t-test.

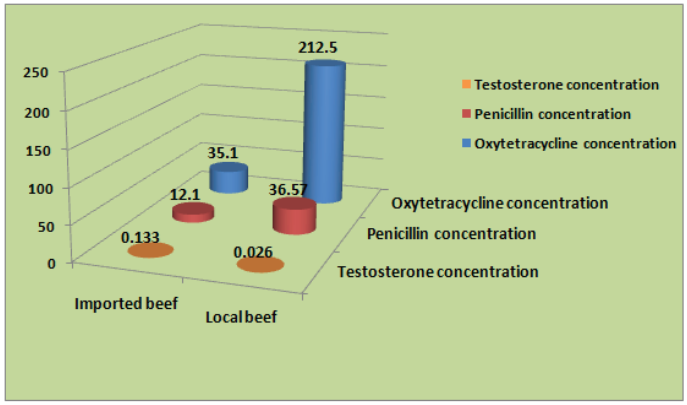

Figure 1: Mean values of oxytetracycline, penicillin & testosterone concentrations (µg/kg) in the examined samples of local and imported beef

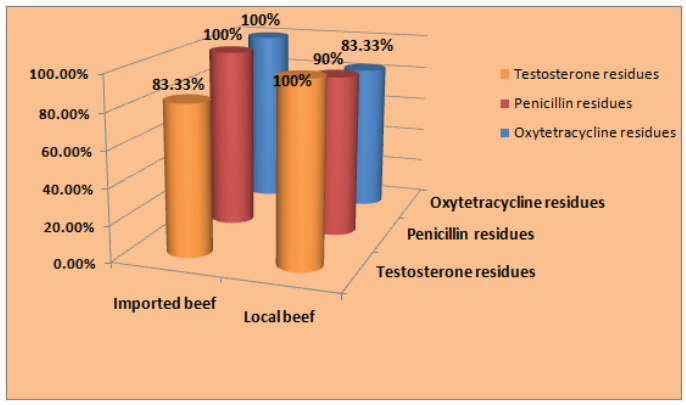

Figure 2: Acceptability of oxytetracycline, penicillin& testosterone residues in the examined samples of local and imported beef

within the permissible limit, in contrast, 10%of the examined local beef samples were unaccepted. The current results come in accordance with those reported by Adesokan et al. [15] in South-Western Nigeria who reported that each of meat specimens contained detectable levels of PEN-G with a mean value of 11.67 ± 2.94 µ/kg .Lower percent was reported by Ibrahim et al. [21] in Nigeria who found that positive samples of PEN-G residues were 14%. It is of interest to note that the examined local beef samples contained detectable residues of either OTC or PEN. G. that might be due to the fact that these two antimicrobials are amongst the drugs most commonly administered by livestock workers in Egypt specially and Africa in general. This finding is in agreement with the recent report from a survey carried out in South Africa (Eagar and Van, 2012) [22]. Where TCs constituted the second largest group of antimicrobials and PEN.G is the majority of the parenteral dosage forms sold.

(Table 3) and (Figure 1) indicated that testosterone residues were detected in 76.67 % and 73.33 % in the examined samples of imported and local beef, respectively. Moreover, the levels of testosterone residues (µg/kg) ranged from 0.014 to 0.605 with a mean value 0.133 ± 0.008 for imported beef and from 0.011 to 0.047, with a mean value of 0.026 ± 0.002 for local beef. The difference between the examined samples of imported and local beef were highly significant (P<0.01).Regarding to FAO/WHO (2004) [23] which recommended that the maximum residual limit of testosterone residue in meat is0.100 µg/kg, so, Table (3) and Figure (2) indicated that 16.67% of the examined imported beef samples were unaccepted. In contrast, the examined samples of local beef were accepted where their

testosterone residues were within the permissible limit .The present results agree with those recorded by Oveisi et al. [24] in Tehran who revealed that the mean concentration level of cattle meat testosterone was 0. 810 uµg/kg . Lower results were reported by Mahgoubet al. [25] in Sultanate of Oman who recorded that the maximum level of testosterone residues in meat samples was 0.05 µg/kg, while higher results were reported by Zeitoun and Ahmed [6] who recorded that the mean value of testosterone residues in meat was 1.8602 ± 0.1148 µg/kg. The use of hormones had been banned in Italy since 1961, in Denmark since 1963, and in Germany since 1977. Belgium and Greece had never permitted the use of hormones for fattening purposes. However, Spain, the United Kingdom, France and Netherlands permitted the use of most hormones for speeding growth in beef cattle [26], so that the examined samples of imported beef meat had high concentration of testosterone residues. The effect of different cooking methods on the oxytetracycline, penicillin and testosterone residues in beef was shown in (Table 4) and (Figure 3) which showed that the mean concentrations of tested control samples of oxytetracycline, penicillin& testosterone residues were 283.78, 45.48and 0.32 µg/kg ,respectively , by application of different cooking methods(boiling ,grilling and microwave) the reduction percentage were 74.54%, 96.9% &77.54%; 90.5%, 98.28% & 96.08 % and 64.74 %, 92.38% & 78.52% for oxytetracycline, penicillin and testosterone residues respectively. The lower results were reported by Zeitoun and Ahmed [6] who recorded that the reduction percentage of testosterone residues were 29%, 23% and 19% for grilling, microwave and oven, respectively. Cooking time and temperature are two main factors which affect antibiotic residues in meat, where in some cooking procedures, sufficient heating temperature and time can reduce several antibacterial drug residues although it does not generally provide an additional margin of safety for consumers [27]. It is concluded that the most preferable methods of cooking for reducing antibiotic and hormone residues on meat were grilling followed by microwave and boiling .To safeguard consumers from the risk of such residues proper use of antibiotics through good diagnosis of the diseases by experienced veterinarians and creation of legislation of compulsory following the rules of withdrawal time of the antibiotic and hormone. Control of antibiotic and hormone residues by heat treatment by cooking processes that have a higher temperature and longer time should be done to inactivate antibiotic and hormone residues in meat and it is

		Imported Beef	Local beef
positive samples	No	23	22
	%	76.67	73.33
Minimum		0.014	0.011
Maximum		0.605	0.047
Mean ± S.E*		0.133 ± 0.008	0.026 ± 0.002++
Maximum Residual Limit (µg/kg)*		0.100	0.100
Accepted samples	No	25	30
	%	83.33	100
Unaccepted samples	No	5	0
	%	16.67	0

Table 3: Statistical analytical results and acceptability of the examined samples of local and imported beef according to their contents of testosterone residues (µg/kg) (n = 30).
++= High significant differences (P<0.01) as indicated by t-test.

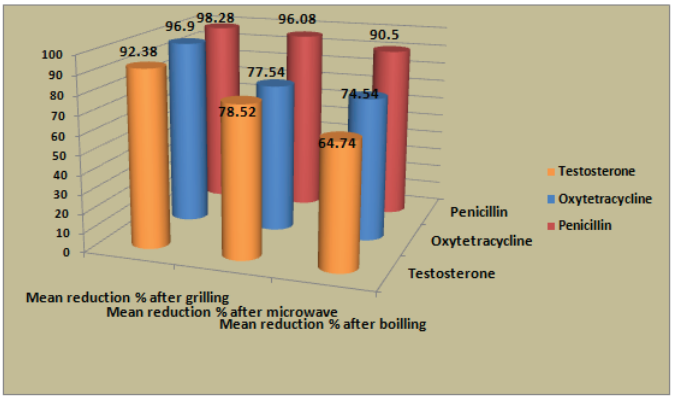

Figure 3: The effect of different cooking methods (boiling, microwave and grilling) on the oxytetracycline, penicillin and testosterone residues in beef (Reduction %).

Trial			1	2	3	4	5	Mean ± S.E*
Control		Oxytetracycline	48.3	198.5	256.1	314.2	601.8	283.78
		Penicllin	10.4	29.7	52.9	63.1	71.3	45.48
		Testosterone	0.012	0.098	0.392	0.489	0.605	0.32
Boiling (100°Cfor 30min.)	Content	Oxytetracycline	0	7.8	69.4	127.5	335.2	107.98
		Penicllin	0	0	2.2	10.6	18.9	6.34
		Testosterone	0	0	0.185	0.297	0.414	0.179
	Reduction%	Oxytetracycline	100	96.1	72.9	59.4	44.3	74.54
		Penicllin	100	100	95.8	83.2	73.5	90.5
		Testosterone	100	100	52.8	39.3	31.6	64.74
Grilling (200°Cfor 15min.	Content	Oxytetracycline	0	0	0	5.3	82.6	17.58
		Penicllin	0	0	0	1.7	4.2	1.18
		Testosterone	0	0	0	0.061	0.155	0.043
	Reduction%	Oxytetracycline	100	100	100	98.3	86.2	96.9
		Penicllin	100	100	100	97.3	94.1	98.28
		Testosterone	100	100	100	87.5	74.4	92.38
Microwave (180°Cfor 10min.)	Content	Oxytetracycline	0	0	13.8	47.1	119.5	36.08
		Penicllin	0	0	0	3.8	9.6	2.68
		Testosterone	0	0	0.099	0.184	0.269	0.110
	Reduction%	Oxytetracycline	100	100	94.6	85	80.1	77.54
		Penicllin	100	100	100	93.9	86.5	96.08
		Testosterone	100	100	74.7	62.4	55.5	78.52

Table4: Effect of different cooking methods on the oxytetracycline, penicillin & testosterone residues in beef.

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advised that meat broth should be discard in order to reduce exposure to residues as the most of antibiotic and hormone residues during boiling procedure excreted from tissue to cooking fluid.

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