Oxytetracycline residues in marketed Frozen beef livers at Sharkia, Egypt

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A B S T R A C T

The present study was performed to determine the presence of antibiotic residues in local and imported frozen liver samples marketed at Sharkia Governorate, Egypt. One hundred local liver samples were examined by microbial inhibition test for antibiotic residues, 5 samples (5%) react positive while all the examined imported frozen liver samples (20 samples) were free from antibiotic residues. Oxytetracycline residues were found in local liver samples with an average of 2.51- 8.78 µg/g (mean 4.81 µg/g) using high performance liquid chromatography (HPLC). All positive samples are above the permissible limit.

KEY WORDS: liver, oxytetracycline, residues

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1. I N T R O D U C T I O N

Liver is the largest organ in the body, possesses a great variety of nutrients and act as an excellent source of high quality animal protein, fat and carbohydrates. In addition, it is a generous supply of certain vitamins and minerals. Liver is easily deteriorated, so it necessitates specific care during handling and preservation to improve its quality and protect human health. The proximate composition of liver as moisture (71.92%), protein (18.44%), fat (5.60%) and carbohydrate (2.72). Mineral concentration (mg %) in liver are sodium (60.04), potassium (274), calcium (5.60), magnesium (6.20), iron (20.86) and copper (5.60) (Tollefson, and Miller, 2000).

Egypt imports a large quantities of frozen beef liver from United States of America and other countries. Many problems lead to hindering the international flow of liver trade and most of them were confined to the shelf-life issue which in the Egyptian standards is seven months. Currently approximately 80% of food-producing animals receive medication for part or most their lives.

Antibiotics are widely used in veterinary medicine in large scales for prophylaxis and treatment of different diseases. Also, they may be used as growth promoters and feed additives (Lee, 2000).

Antibiotic residues and their metabolites are pharmacological active substances as they are remain in food stuffs above Maximum Residue Level (MRL). WHO and FAO establish tolerances (MRL) for drugs in the relevant tissues of food producing animals, the tolerance is the tissue concentration below which a marker residue for the drug or chemical must be fall in the target tissue (Nisha, 2008).

Joint FAO/WHO Expert Committee on food additives (Reig, and Toldra, 2008) recommended the residues of some veterinary drugs in animal tissues and foods and determined the MRL for Oxytetracycline as 200 µg/kg, 600 µg/kg

and 1200 µg/kg for beef, liver and kidney, respectively.

The Maximum residue limits (MRLS) which tolerated by (EU Council Directive 96/23/EC). Determined the Maximum residue limit (MRL) for tetracycline as follow: 100 µg kg-1, 300 µg/kg, and 600 µg/kg for muscle, liver and kidney, respectively.

Permissible limit for the drug is the value or the average at which tissue concentration of the drug is safe for human consumption. Subsequently drug residues may persist in foods derived from animals which may pose an adverse health effect for the consumer. Antibiotic residues may lead to allergic reactions, imbalance of intestinal microflora and antibiotic resistance which may cause treatment failure or the need to overuse of more toxic drugs because of increasing frequency, duration or severity of infection. Antibiotic resistant strains of bacteria known to be food-borne pathogens such as Salmonella spp, E. coli, and Campylobacter spp. have been isolated from farm animals (Aryal, 200, Emborg, et al., 2003, Belloc, et al., 2005). Potential adverse effects form antibiotic residues in meat threaten human health lead to acute toxicity, carcinogenicity, reproductive effects and allergic reactions (Gehring, and Riviere, 2006).

Withdrawal periods and residue control are conducted in slaughter houses to prevent harmful drug residues in food of animal origin that consumed by human being (McEwen, and Fedorka-Cray, 2002).

Different methods usually used to detect antibiotic residues in food of animal origin as microbiological, immuno-enzymatic and chemical, A screening method is the first-hand analysis of the sample to establish the presence or absence of residues and the microbiological type is suitable for large scale screening because of their convenience and broad spectrum characteristics (Aerts, et al., 1995). High performance liquid chromatography (HPLC) is widely used to quantify various antibiotic residues in food products with good sensitivity and specificity (Muriuki, et al., 2001).

The aim of the present study was to investigate the qualitative detection of antibiotic residues in random marketed local and frozen liver samples by using the microbiological assay technique.

In addition to the quantitative determination of oxytetracycline residues in examined samples was applied by using high performance liquid chromatography (HPLC).

2. MATERIALS AND METHODS

2.1. Collection of samples:

100 Local fresh and 20 imported frozen meat samples were randomly collected from meat shops, supermarkets at Sharkia Governorate, Egypt. Each sample was transferred in a separate sterile and labeled plastic bags in an ice-box to the laboratory without undue delay, therefore, all samples were analyzed for determination of oxytetracycline residues.

2.2. Detection of Oxytetracycline residues in meat samples:

2.2.1. Microbial inhibition test:

The technique of Levetzow and Weise (1979) is adopted for detection of antibiotic residues in liver samples (Levetzow, and Weise, 1979).

2.2.1.1. Preparation of the spore suspension:

Nutrient agar was inoculated with heavy suspension of Bacillus subtilis bacteria and is incubated at 30° C for 10 days. The growth was harvested with a sterile physiological saline (0.8% Sodium chloride), then centrifuged at 3000 r.p.m. for 10 minutes. The supernatant fluid was discarded and 10 ml of sterile

physiological saline was added to the sediment. After mixing, the suspension was centrifuged for 10 minutes at the same speed them the supernatant fluid was removed and the process was repeated twice. The test suspension was heated in a water bath at 70° C for 30 minutes then diluted with normal physiological saline to achieve a density of 107 spores/ml of diluents by comparing with Mcfidian tube.

2.2.1.2. Preparation of test plates:

The media was adjusted at pH 6.0 for detection of tetracycline residues. The medium was left to cool to 55° C, and then it was inoculated with the spore suspension of Bacillus subtilis (0.1 ml/100 ml medium). The ingredients was poured on Petri dishes and left for complete solidification and when test plates were not used immediately, they were stored in a refrigerator below 4° C for a maximum period of one week.

2.2.1.3. Maintenance of B. subtilis culture:

Tubes containing slopes of nutrient agar were inoculated with Bacillus subtilis and were incubated overnight at 30° C. The culture were stored in refrigerator at 4° C and re-inoculated every month.

2.2.1.4. Sample treatment:

The superficial layer of the frozen samples was removed by a sterile scalpel. Cylindrical pieces were removed from the core of the samples using a sterile cork borer (internal diameter 8 mm). Slices 2 mm thickness of the cylindrical pieces were put diagonally on the Bacillus subtilis plates. The sensitivity of all test plates was monitored by applying 6 mm diameter filter paper discs impregnated with sodium benzlpenicillin (0.01 i.u./disc) and streptomycin (0.5 ug/disc) which gave annular zones of inhibition of a specified minimum size, that is ≥ 6.8 mm extending from the discs. All plates were incubated at 30° C for 18-24 hours and then the zones of inhibition were measured.

2.2.1.5. inhibition zones:

The result was indicated by measuring the diameter of inhibition zones of the growth of the Bacillus subtilis cells around liver samples. A zone more than or equal to 2 mm was recorded as positive result, however, zone from 1 to 2 mm was recorded as suspicious one and a zone less than 1 mm was considered as negative result.

2.3. High performance liquid chromatography (HPLC) method (Heitzman, 1994):

The liver samples determined as positive by microbiological assay were further analyzed by HPLC for identification and quantification of oxytetracycline residues. Chromatography is a technique used for separating mixtures into their individual components, so that they can be identified and measured.

2.3.1. chromatograply:

In liquid chromatograply (LC), a moving liquid (the mobile phase) carries the sample across a stationary phase (the solid support found within a LC column). The sample components separate based on their differing affinity with stationary phase. Every liquid chromatography usually includes the following key components: a pump system for solvent delivery, a sample injector, a column or columns, detectors and a data hadling system.

2.3.2. Analytical method:

2.3.2.1.Extraction of the drug from sample.

Frozen liver samples were thawed and finely diced with scissors after trimming of the external fat and fascia.

Two grams of each organ to be analyzed were weighed using digital balance and then cut into very small pieces and subsequently ground into fine powder using Sartorius mincer. This was then homogenized in a blender for 2 min. and then 0.1 gm 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 um nylon filter, 20 ul of solution was injected into HPLC for analysis according to (Senyuva et al., 2000).

2.3.2.2.Chromatographic condition:

Included a 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. The separation was done on Hypersil gold C18 (10um, 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).

2.3.2.3.Calibration curve:

The curve was prepared by using concentrations of 0.01, 0.1, 0.5, 1.25, 2.5, 5, 10, 20, 50 mg/L of OTC 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 3.7 minutes

3. RESULTS

Incidence of oxytetracycline residues by microbial inhibition test in examined 100 samples of local beef liver and 20 imported frozen liver samples show that 5 samples of local liver are unfit (5%) while, all imported frozen samples are normal and free from any residues (Table 1).

The size of inhibition zone in mm for positive local liver samples using microbial inhibition test are ranged from 3mm to 5 mm (Table 2).

Oxytetracycline concentration in positive local liver samples determined by HPLC method show increase from the permissible limit which is 0.3 µg/ gm in all samples that the concentrations are ranged from 2.505509 µg/ gm in the sample number three till 8.779393 µg/ gm in sample number one (Table 3).

4. DISCUSSION

Oxytetracycline (OTC) is a broad-spectrum antimicrobial agent that is active against bacteria and some chlamydiae, rickettsiae and protozoa. It is widely used in veterinary medicine because of its wide spectrum and advantageous pharmacokinetic features (Mestorino, et al., 2007).

Oxytetracycline is widely distributed into body tissues and can be found in high concentration in the excretory organs especially the liver and in the bile (Prescott, and Baggot, 1993). Withdrawal time of oxytetracycline must be known for deciding date slaughtering (Riviere, 1991).

Incidence of oxytetracycline residues in local and imported frozen liver samples was detected by microbiological assay. Out of 100 examined local liver samples, 5 (5%) react positive for antibiotic residues, which may indicate the misuse of antibiotics by veterinarian and owners as well as the absence of monitoring of antibiotics residues in slaughterhouses as recorded in table (1).

Higher results were obtained by (Muriuki, et al., 2001, McEvoy, 2002, Shahid, et al., 2007, Abasi, et al., 2009, Olufemi, and Agboola, 2009, Donkor, et al., 2011, Wahab allal, et al., 2011), while lower results were obtained by (Marouf, and Bazalou, 2005, Biswas, et al., 2007, Presi, et al., 2007).

Similar results reported by (Shahid, et al., 2007) and lower result were obtained by (Wahab allal, et al., 2011).

Oxytetracycline tissue concentration (ug/g) after intramuscular administration was found in muscle (injected site), kidney and liver at 1st and 2nd week after administration but not found in kidney at 3rd and 4th week while was found in liver and injected site (Mestorino, et al., 2007).

The level of antibiotic residues was highest in liver samples. This is due to the role of the liver in the metabolism and detoxication of most antibiotics by its microsomal enzymes which may lead to high levels of antibiotic residues through its tissues (Tajik, et al., 2010, Salama, et al., 2011).

The results recorded in table (3) revealed that the use of HPLC for detection of oxytetracycline were 8.779393, 3.22068, 2.505509, 5.557753 and 3.976435 for all positive samples and these results are above the permissible limit. Lower incidence was reported by (Marouf, and Bazalou, 2005, Presi, et al., 2007). Moreover, none of the examined 20 frozen liver samples were reactive positive for oxytetracycline residues, and these results were similar to those reported by (Gehad, 2002, Mahmoud, and Mohsen, 2008) whom reported that there is no antibiotic residues could be detected in cattle muscle and organs after freezing for a long period. Slightly higher results were obtained by (Myllyniemi, et al., 2000) who examined samples taken from emergency slaughtered animals during the withdrawal period of an antibiotic treatment and found that 68 % out of 89 samples containing residues and revealed a wide range of penicillin, oxytetracycline and enrofloxacin concentrations.

results showed in table (3) revealed that use of HPLC for detection of oxytetracycline were 8.779393, 3.22068, 2.505509, 5.557753 and 3.976435 and these results are above the permissible limit. Tetracyclines occasionally associated with peripheral blood changes, discoloration of bones and teeth due to binding with calcium ions and allergic reaction in humans. They added that low doses (20mg oxytetracycline per person daily) might have some impact on the fecal anaerobic microflora of human. This observation was used to establish an allowable daily intake (ADI) for human being was 0.003 mg/kg (David, and Scott, 1994, Wlatner-Tows, and McEwen, 1994, Muriuki, et al., 2001., Samanidou, et al., 2007).

In Egypt ,the overuse of antibiotics in veterinarian field lead to high quantities of antibiotics residues in liver which that affects animal health and lead to public health hazard while mean frozen livers imported from foreign (advanced) countries have great restrictions on antibiotic residues. In addition, freezing usually destructs antibiotics, so there were no antibiotic residues in frozen liver samples.

Table 1 Incidence of oxytetracycline residues by microbial inhibition test in the examined local and imported frozen liver samples.

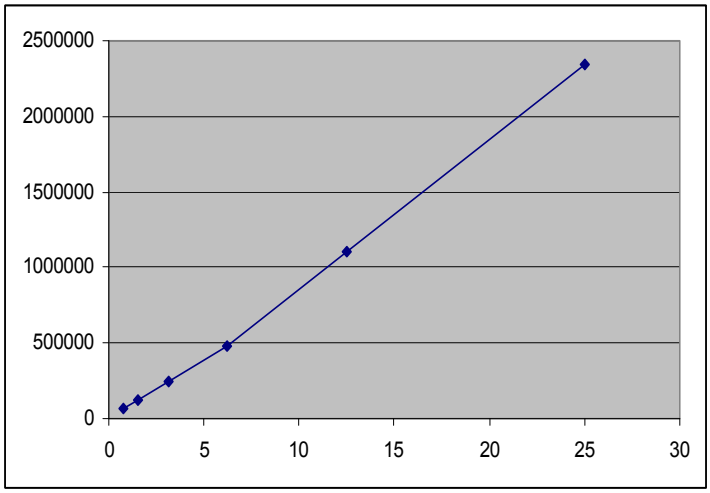
Samples	No. of examined samples	Unfit Samples	
		No.	%
Local liver	100	5	5 %
Imported frozen liver	20	-	-

Table 2. The size of inhibition zone in mm for positive local liver samples using microbial inhibition test

Liver Samples	Inhibition Zone
No.1	3
No.2	4
No.3	3.5
No.4	5
No.5	3

Table 3 Oxytetracycline concentrations in positive local liver sample determined by HPLC method

Sample	Oxytetracycline concentrations (µg / gm)	Permissible limit (µg / gm)
No.1	8.779393	0.3
No.2	3.22068	0.3
No.3	2.505509	0.3
No.4	5.557753	0.3
No.5	3.976435	0.3



Chromatogram of oxytetracycline standard (6.25 µg /ml)

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بقايا الأوكسيتتراسيكلين فى أكباد الأبقارالمجمده المسوقة بالشرقية، مصر

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الملخص العربى

أجريت هذه الدراسة لمعرفة مدى وجود بقايا الأوكسي تتراسيكلين فى اكباد الابقار المجمده المحلية وأيضاً المستوردة، حيث تم تجميع 100 عينة من الأكباد المحلية و 20 عينة من المستورده من محلات الجزارة والسوبر ماركت المختلفة من مدينة الزقازيق – محافظة الشرقية بجمهورية مصر العربية. وقد أسفرت النتائج وجود هذا المضاد الحيوى فى 5 عينات من عينات الأكباد المحلية باستخدام الفحص الميكروبيولوجى و الكمي بجهاز HPLC كما لوحظ خلو الأكباد المجمدة المستوردة منها وبعد فحص بعض العينات المحتوية على بقايا هذا المضاد الحيوى بواسطة الفحص الكمي عن طريق جهاز HPLC ، وجد أن عينات الأكباد المحلية قد تعدت النسب المسموح بها دولياً حيث وجد بمتوسط 4.81 ميكروجرام/ جم ولهذا نوصى بأتباع التعليمات الخاصة بكل عقار عند استخدامة فى علاج الحيوان قبل الذبح وذلك للحفاظ على صحة الانسان.

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