

Detection of Oxytetracycline Residues in Table Eggs in Khartoum State, Sudan

ABSTRACT

Aims: This study was conducted to detect the presence of oxytetracycline residues in table eggs in Khartoum State and to compare its level with the international acceptable maximum residue limits (MRLs).

Study design: One hundred and eighty table egg samples were randomly collected from 18 sale points in the three localities of Khartoum State, Sudan, (60 eggs from Omdurman, 60 eggs from Khartoum and 60 eggs from Khartoum North).

Place and Duration of Study: samples collected from Khartoum State during August and September, 2015.

Methodology: Microbiological inhibition assay was used to screen the presence of antibiotic residues using *Bacillus subtilis* seeded in nutrient agar. Ninety positive egg samples from the microbiological inhibition assay were analyzed to detect the presence and quantify of oxytetracycline residues using HPLC.

Results: Microbiological inhibition assay showed that 50% of the tested samples were positive for antibiotic residues in Omdurman, Khartoum and Khartoum North with 34(18.9%), 28(15.6%), and 28(15.6%) of the antibiotics respectively. HPLC results showed that 63 (70%) were positive for oxytetracycline residues 21.2 % from Omdurman, 23.3 % from Khartoum and 25.5 % from Khartoum North.

Conclusion: It was concluded that high percentage of table eggs contained oxytetracycline residues above the MRLs (0.2ppm) that indicated the widespread misuse of oxytetracycline in poultry farms that may cause health hazards to consumers in Khartoum State. Therefore the study recommends compliance of drug withdrawal periods in poultry farms could reduce the incidence of antibiotic residues in consumed eggs.

(Keywords: oxytetracycline. residue, table eggs, health, microbiological, HPLC, analysis)

1. INTRODUCTION

Birds are reared for meat and egg production as an important protein sources. Drugs are an essential part of poultry production and used to prevent and control diseases, reduce stress due to environmental changes, vaccination and other management practices [1]. Recently in Sudan, the poultry industry has been growing. The use of antibiotics in routine poultry production has become unavoidable in order to prevent economic losses due to poultry diseases [2].

The frequent use of antibiotics for livestock disease prevention causes the occurrence of antibiotic residues in various food products of animal origin including milk, egg, and meat [3]. The presence of antibacterial drug residues in eggs can pose a health hazard to consumers such as hypersensitivity reaction, development of resistant organisms to these antibacterial agents and destruction of gastrointestinal natural microbiota [4]. Consumers in Khartoum state constantly face the risk of exposure to antimicrobial residues in table eggs [5]. Oxytetracycline (OCT) is a broad spectrum antibiotic against Gram-positive and Gram-negative bacteria. It is used for the treatment of bacterial diseases and most commonly applied among tetracyclines group for food producing animals. OCT also finds application in human therapy [6]. It is widely administered to farm animals for the purpose of health protection to prevention of diseases [7]. Worldwide, national and international public health agencies have a deep concern about the presence of antibiotic residues in meat, egg and edible viscera of food producing animals [8, 9]. The maximum residue levels (MRLs) for veterinary drugs are implemented by the European Union and similarly suggested by other international institutions to prevent these problems and safeguard consumers' health [10, 11]. Oxytetracycline residues was detected in table eggs in studies conducted by Alghamdi [12] Naser [13]. The European Union has established the maximum residual limit (MRL) of oxytetracycline in eggs at 0.2ppm [10]. This study was aimed to detect the presence of oxytetracycline residues in table eggs in Khartoum State and to compare its level with the international acceptable maximum residue limits (MRLs).

Comment [S1]: It is not "was". It is "were" because you are referring to the oxytetracycline residues that are many residues

2. MATERIAL AND METHODS

2.1 Sample collection

A total of 180 table egg samples were collected randomly from 18 sale points (farms) in the three major localities in Khartoum State, (60 eggs from Omdurman, 60 eggs from Khartoum, 60 eggs from Khartoum North) between August and September, 2015. The source of the samples to the sale points were 18 farms.

Comment [S2]: The sentence should be eliminated, because this information is mentioned to the beginning of the paragraph

52 2.2 Sample handling and processing

53 The egg samples were transported to the Central Veterinary Research laboratory in carton and plastic storage
54 trays at room temperature (22-25°C) and processed within 24 hours post collection. The surface of each egg
55 was disinfected using sterile cotton soaked in 70% ethyl alcohol in a cabinet (under septic conditions). A small
56 crack was made at the tip of the egg using a sterile thumb forceps, after which the albumen and yolk were
57 carefully mixed using a sterile cotton swab, which was subsequently removed into a sterile universal container
58 and preserved at -20°C until analyzed.

59 2.3 Evaluation of antibiotic residues

60 Two methods were used simultaneously for the determination of the presence oxytetracycline residues in
61 table eggs namely screening test that included microbiological inhibition assay and confirmatory test using
62 high performance liquid chromatography (HPLC).

63 2.3.1 Microbiological inhibition assay

64 *Bacillus subtilis* (ATCC6633) was obtained from the Department of Bacteriology, Central Veterinary Research
65 Laboratory, Khartoum, Sudan and used as an indicator organism according to the method described by
66 Ellerborek [14] with some modification.

67 2.3.1.1 Preparation of bacterial suspension

68 A sterile loop was used to transfer some colonies of *Bacillus subtilis* previously grown on nutrient agar slant,
69 onto a nutrient agar plate and incubated at 37°C for 18 hours. One colony from the fresh culture was taken by
70 using sterile loop and diluted in 5ml sterile 0.85% NaCl the suspension was mixed by vortex mixer then
71 adjusted to 0.5 in a McFarland tube.

72 2.3.1.2 Preparation of culture medium

73 Nutrient agar medium (Oxoid) was prepared according to the manufacturer instructions then cooled to 45-50
74 °C. 10µL (1.5×10⁶ CFU) of the bacterial suspension was taken by micro pipette and inoculated into the medium
75 then mixed gently and poured into sterile Petri dishes in 25ml volume and left to solidify.

76 2.3.1.3 Test procedure

77 Four wells were made in each petri dish by a sterile puncher. 0.25g from the albumin and yolk mixture was
78 taken using a sterile syringe, inoculated into the wells then incubated aerobically at 37°C for 18 hours. The
79 presence of antibiotic residues in the sample was indicated by the appearance of inhibition zone around the
80 wells.

Comment [S3]: It should be "Petri" and not "petri"

81 **2.3.2 .HPLC analysis**

82 HPLC analysis was used for the determination of oxytetracycline residues levels in 90 positive samples that
83 tested positive using the microbiological inhibition assay.

84 **2.3.2.1 Materials**

85 Oxytetracycline and tetracycline reference standards were obtained from Dopine Department, Pendik
86 Institute, (Republic of Turkey). Citric acid, HPLC grade acetonitril (ACN) and methanol were obtained from
87 Sharlau (company in Germany), nitric acid 30%, deionized water and sulfuric acid was obtained from Sharlau
88 (company in Germany).

89 **2.3.2.2 Preparation of oxytetracycline standard stock and working solutions**

90 Stock solution of 1mg/ml of oxytetracycline was prepared by dissolving 0.01g of oxytetracycline standard in
91 100ml methanol HPLC grade in a volumetric flask. The working solutions of 200, 100, 50, 25 µg/ml was
92 prepared by addition of 200, 100, 50, 25 µl of stock solution methanol to be completed to one ml each
93 concentration respectively.

Comment [S4]: It should be "were"

94 **2.3.2.3 Preparation of tetracycline standard stock**

95 Stock solution of 1mg/ml of tetracycline standard was prepared by dissolving 0.01g tetracycline in 100 ml
96 methanol then preserved at 4°C in a fridge. Tetracycline was used as internal standard.

97 **2.3.2.4 Preparation of mobile phase**

98 Fifteen ml of acetonitrile (ACN) was added to 85 ml of deionized water (pH = 2.1) with sulfuric acid (15:85)
99 made up to 100 ml. Fresh mobile phase was prepared daily before work.

100 **2.3.2.5 Sample extraction**

101 Egg samples were extracted by liquid – liquid extraction according to method described by Senyuva [15]. Two
102 grams of homogenized egg sample were taken and transferred into centrifuge tube, 100µL (100µg/ml) of

tetracycline was added as internal standard, 0.1 g citric acid, 1ml nitric acid (30%), 4 ml methanol and 1 ml deionized water were added respectively. The suspension was vortexed, kept in ultrasonic water bath for 15 min and then centrifuged at 5300 rpm for 10 min. the supernatant was taken and filtered through a 0.22 µm syringe filter then 20 µL of solution was injected into HPLC for analysis.

2.3.2.6 Preparation of standard curve

Calibration standards were prepared using concentrations of 25, 50, 100 and 200 µg/mL of oxytetracycline. Drug spiked egg sample were prepared by adding appropriate amount of stock solution to two gram of blank egg sample to make a final concentration of 25, 50, 100 and 200 µg/kg

Validation of the method

The linearity of the methods was tested in egg over the range of 25, 50, 100 and 200 µg/ml. the precision of the method was evaluated by calculating relative standard deviation (RSD) in intraday repeatability. RSD calculated from three replicate for each spiking level.

2.3.2.7 HPLC determination of oxytetracycline residues concentration

Determination of oxytetracycline residues was performed by using a HPLC system (Cykam, Germany) and S3210 uv-vis detector, Cykam S1122 quaternary pump and column type Interstil ODS-3 C18 (5 µm, 150 X 4.6 mm). The detecting wavelength was set at 360 nm and personal computer software (simple peak) was used for analyzing data. The mobile phase contained deionized water (pH = 2.1 with sulfuric acid) (A) and ACN (B). Isocratic solvent program was run (85 % (A): 15 % (B), v/v). The Flow rate was 1.5 ml/min. HPLC analysis of the samples was performed in 10min. Peak area ratios of oxytetracycline were converted to concentrations using the calibration curve.

3. RESULTS AND DISCUSSION

3.1 Microbiological inhibition assay

Of the 180 egg samples 90 (50%) showed the presence of antibiotic residues and 90 (50%) were negative. The total number of positive and negative samples which were found in samples collected from Omdurman, Khartoum and Khartoum North are shown in Figure 1.

Comment [S5]: This information is not indispensable. But if the authors want to include it, they should make it like part of the previous paragraph and don't it as an independent aspect.

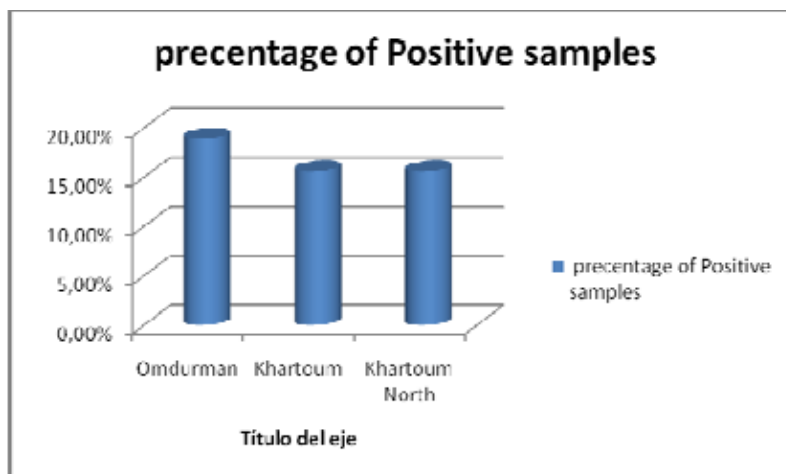


Figure 1: Percentage of positive egg samples for antibiotics residues using microbiological inhibition assay in Omdurman, Khartoum and Khartoum North.

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3.2 HPLC analysis

Of the 90 Positive samples for antibiotic residues in Khartoum State, showed that 63 (70%) were positive for the residues above the permissible maximum residue limits and 27 (30) % were below (Figure 2). The mean concentration of oxytetracycline residues in positive eggs were 24.1 ppm in Omdurman, 24.7 ppm in Khartoum and 30.6 ppm in Khartoum North respectively.

3.2.1. Result of validation of method

The standard curve showed good linearity over the range of concentrations examined (25-200µg/ml) where a straight line passing through the origin with a correlation coefficient of R^2 ; 0.9965 was obtained for oxytetracycline (Figure 3). All RSD % value for intraday repeatability ranging from 1.40 - 7.40.traeness expressed as relative recovery 73.9 - 92%.

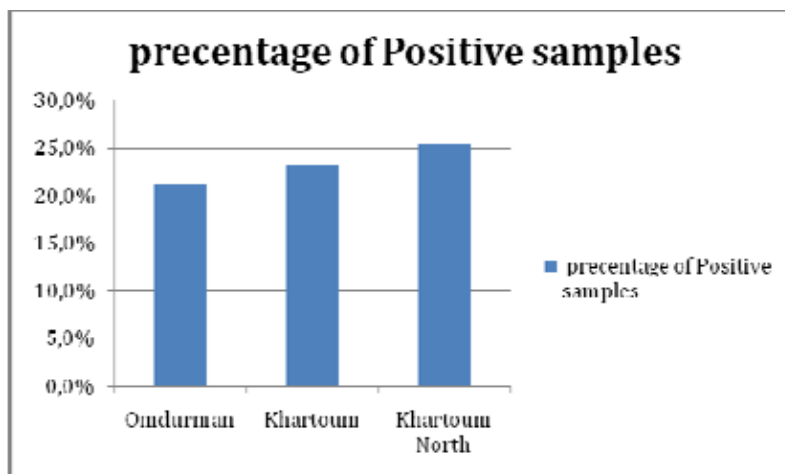


Figure 2: The percentage of positive egg samples for oxytetracycline residues using HPLC in Omdurman, Khartoum and Khartoum North.

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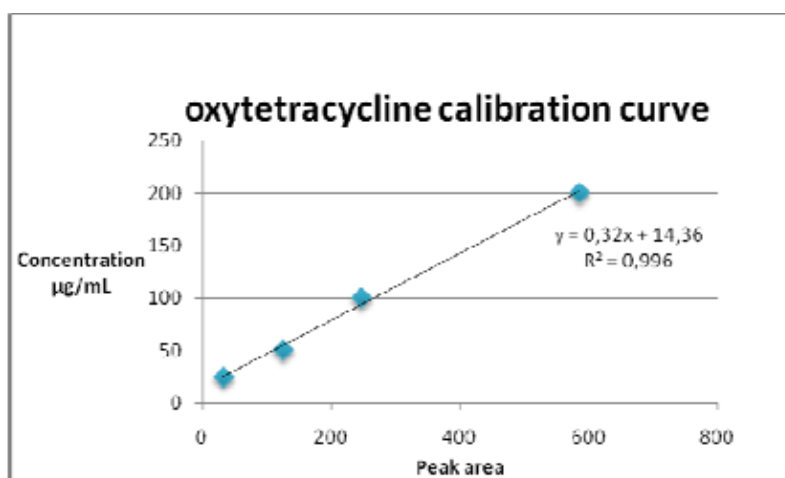


Figure 3: Oxytetracycline calibration curve.

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Table eggs in Khartoum State were screened for the presence of oxytetracycline residues by using microbiological inhibition assay and HPLC. The microbiological method can detect a wide range of antibiotics thresholds close to the maximum residue limits (MRLs) [1,16]. The results of present study revealed that 50% of egg samples were positive for antibiotic residues in 16 sale points in the three localities in Khartoum State. Higher percentages of positive samples for antibiotic residues were observed in Omdurman 18.9% than Khartoum 15.6% and Khartoum North 15.6%. Nearly similar results were reported in study done in Khartoum

State [17, 5]. Our results showed that table eggs in sale points were contaminated with antibiotic residues that may expose consumers to health hazards. This also indicated the widespread misuse of antibiotics which may be as a result of poor hygienic status of poultry farms necessitating the continuous use of antibiotics for treatment of diseases and prophylaxis purpose. It was also proposed that lack of knowledge about observing the withdrawal period following the use of antibiotics resulted in the high percentage of positive samples [17]. Samples positive for drug residues were further analyzed for the detection and quantification of oxytetracycline residues using high performance liquid chromatography method and results revealed that 70% were positive for oxytetracycline residues above the permissible maximum residue limits according to European Union in the 16 sale points in three localities in Khartoum State. Higher percentages of positive samples for antibiotic residues were observed in Khartoum North 25.5% Khartoum 23.3% Omdurman 21.1%. Some positive samples for microbiological inhibition assay showed negative results for oxytetracycline residues for HPLC may be due to variation of antibiotic use by different farms. This was in line with other investigators (Hind *et al.*, 2012) who reported that the most frequently used antibiotic was tetracycline in 36.4% of the farms. The observations in the present study are similar to the findings reported by Alghamdi *et al.* (2000) who found higher usage of tetracycline in eggs in Saudi Arabia using the high-performance liquid chromatography (HPLC) and Naser (2012) who found positive samples with seasonal differences in different areas. These results showed a strong contamination of the table eggs samples with oxytetracycline residues that may indicate wide usage for oxytetracycline and widespread misuse by breeders (poultry) in Khartoum State.

Comment [S9]: It is important to include the reference "(European Union Regulation, 2009)"

4. CONCLUSION

In conclusion, this study has shown that a high percentage 63(70%) of table eggs from sale points in Khartoum State contain oxytetracycline residues above the MRL that may indicate the widespread misuse of oxytetracycline in poultry farms which can result in serious health hazards.

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