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**Research Article**

# Detection of Antibiotic Residues in Poultry Tissues in Northern Bahri-Khartoum State-Sudan

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**Abstract:** This study was conducted in Khartoum North – Sudan to detect antibiotic residues in different poultry tissues (liver, gizzard and muscle) using *Bacillus subtilis* as a test organism. A total of 213 tissue samples were obtained from a poultry slaughter house used to slaughter poultry from different poultry farms in Khartoum north. The filter paper and whole tissue methods were used and compared using *Bacillus subtilis* as test organism . The total number of positive samples was 67 (31.4%) The highest percentage of positive samples was detected in the liver tissues 44 (62%) and the least was in the muscles tissue 8 (11.3%). ( ≥ 3mm inhibition zone was regarded positive).There was no significant difference between this standard inhibition zone and the inhibition zone diameter produced by liver samples , but the diameter of the inhibition zone in the gizzard and the muscle vary significantly with the standard inhibition zone set for the test, with p 0≤.05. Kappa statistic was used to measure the agreement between the two method used to detect the antibiotic residues, there was a significant agreement between the two methods with p ≤.05 and the percentage agreement was 78.8%. Also chi square test showed a highly significant association between the two methods. The current study concluded that the poultry meat showed detectable levels of antibiotic residues which may indicate the widespread misuses of antibiotic in poultry farms and the lack of awareness of farmers regarding the recommended withdrawal periods of drugs.

**Keywords:** *Bacillus subtilis*, inhibition zone, antibiotic



## INTRODUCTION

Antimicrobial residues in poultry tissues include a large number of different compounds, which can be administered either in feed, in drinking water or by injection (Reig *et al.,* 2008). Antimicrobial residues in food disrupt the intestinal microbiota and increase the development of resistant bacteria in the general population (Singh S, *et al.,* 2014). Ingesting residues of drugs or their metabolites in meat and other foods of animal origin may cause adverse effects which include; carcinogenicity, mutagenicity, bone marrow toxicity (Chloramphenicol) and allergy (Penicillin) (Nisha. A, 2008).

Poultry farmers uses antibiotics for disease prevention and as growth promoter to increase the rate of feed assimilation and to lower the incidence of mortality caused by a pathogen attack. (Khurram *et al.,* 2018). Antibiotics may result in dysfunctionality of beneficial gut microbiota and increase resistance among microbial pathogens in poultry ( Viviana Clavijo 2018). Residues of these antibiotics in poultry meat and other tissues have been determined in many of the studies globally and are considered one of the possible causes of antibacterial resistance in human pathogens (Viviana Clavijo 2018). The presence of residues of antibiotics in poultry meat and meat products beyond maximum permissible limits is a matter of serious concern (Nisha .A, 2008) Heat treatments can reduce the risk of some sulfonamides, tetracyclines, and fluoroquinolones but do not guarantee the complete elimination or degradation of these antibiotic residues present in broiler products (Khurram *et al.,* 2018).

Drug resistance has gained its importance due to its ability of transmission to other enteric pathogens which have posed serious public health problems (Singh *et al* 2016). In addition soil microbiota which receives antimicrobial residues (AMR) via birds manure may affect the human health as a source of developing resistant microorganisms (Tajick MA and Shohreh B 2006). The Codex Alimentarius Commission (CAC) established the Codex Committee on Residues of Veterinary Drugs in Food which defined Maximum Residue Limits (MRL) for about fifty nine veterinary drugs (Jechalke *et al.,* 2014). Maximum Residue Limit means the maximum concentration of residue resulting from the use of a veterinary medicinal product which may be accepted by the community to be legally

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permitted or recognized as acceptable in or on a food (Codex Alimentarius2014).

Antimicrobial residues are detected by chemical, biological and immunological methods. Detection methods can be classified by their degree of quantification into qualitative, semi-quantitative and quantitative methods. Two types of analytical methods are commonly used, the screening methods that includes microbiological tests (Disc assay, modified Premi and Delvotest methods) (Singh S, *et al.,* 2014 and Tajick MA, Shohreh B 2006) and the confirmatory methods using more complex and advanced techniques such as ELISA and HPLC (Adil. M. Salman, *et al* , 2012)The disc assay and tissue methods are inexpensive and can cover entire antibiotic spectrum but are less specific than other tests (Adil.M. Salman, *et al.,* 2012).

Chicken or poultry meat is largely consumed in Sudan by consumers of all ages and social groups but other bi products such as kidneys, liver and gizzards were consumed mainly by the people in the peripheries for their low prices (Adil.M. Salman, *et al.,* 2012). The use of antibiotic as a routine practice for both prevention or treatment is non avoidable to prevent economic loss due to diseases and consequent mortality. Some of the developed countries, including Sweden, Norway, Denmark, and the European Union have already prohibited the application of antibiotics for prevention, as well as growth-promoting purposes. (Khurram Muaz *et al* 2018). No routine screening programme for slaughtered animals is practiced in the country. Currently, chicken production in Sudan is increasing largely due to increased demand for chicken meat and eggs, more materials and capital farmers venture into the chicken projects but still.

The main objectives of this study was to detect the antibiotic residues in poultry tissue and to compare between the inhibition zones produced by the whole tissue method and the disc assay method.

## MATERIALS AND METHODS

Cross sectional study was carried out to collect samples from Lean slaughter house in Khartoum north in which farmers from different areas of Khartoum North were used to slaughter their birds, A total of 213 samples were collected during August to October 2019 . Birds slaughtered were brought from different farms in Khartoum North. Five 5 grams of chicken tissues (liver ,gizzard and muscles) 71 liver tissue samples, 71 gizzard tissue samples and 71 muscles tissue samples were then collected and transported in thermal box to the laboratory of the college of veterinary medicine in Alkadro.

A questionnaire was designed and filled to gather information from the different poultry farms in Khartoum north which uses this slaughterhouse during June – August, 2019. A total of 8 farms were included in this questionnaire. The general hygienic status of the farms, signs of diseases, the frequency of antibiotics usage, and the awareness of the effects of antibiotic residues by the farmers were investigated.

**Preparation of the disc**

Paper discs were prepared by cutting the filter paper (Whatmann No.1) to the size of approximately 4 mm in diameter and were sterilized.

**Disc Assay Method**

Sterilized nutrient agar plates were inoculated with a loop of freshly prepared suspension of *Bacillus subtilis* . An incision was made in each tissue sample. Then, using sterile forceps, a sterile paper disc was placed and left for few minutes until it was soaked, followed by the transfer of disc to the agar surface. Then the plates were inverted and incubated at 37°C for 24 hours. (Fagbamila *et al.,* , 2010) The diameter of inhibition zone was measured .The inhibition diameters more than 2mm was considered as positive to antibiotic residue (WahabAlla *et al* 2011).

**Whole Tissue Method**

The whole tissue method was done by cutting the samples part to small pieces (1cm in diameter), and placed directly in the nutrient agar plates cultured with *Bacillus subtilis*. Then incubated at 37°C for 24 hours the results were observed to determine the diameter of inhibition zone (Tajik *et al.,* 1998).

**Data Analysis**

Statistical analysis was applied using Statistical Package for Social Sciences (SPSS, version 20.0). Descriptive statistic was used to calculate the frequency and percentage of antibiotic inhibition zones. Univariate analysis using 2-tailed chi-square test was conducted to test the association between the obtained results in different tissues with P-value $\leq$ 0.05 was considered statistically significant. ANOVA was used to test the limits of the diameter of the inhibition zone achieved by the two methods. Kappa statistic was conducted to judge the agreement between the two methods used.

## RESULTS

A total of 213 samples were collected from liver, gizzard and muscles (71 sample of each) to detect antibiotic residues. Eight questioners were filled by the farmer or their representative while in the slaughterhouse.

The regular manure removal was practiced in all farms. The most common sign of diseases was the respiratory signs 75% followed by diarrhea. The most frequently used antibiotic was tetracycline alone in 27.5% of farms, while 62.5% of the farms uses a combination of tetracycline and tylosine or penicillin. The data showed that all of the farms were visited by veterinarians (on call), but 50% of the farmers

administer the drugs by themselves while in the other 50% the veterinarian follow up with the administration of the drugs . 75% of the farms uses antibiotics for only treatment while 25% uses antibiotics for disease prevention and for treatment. 75% of poultry farmers believed that there was no relationship between administration of antibiotics and its appearance in food material and all of them lacked the necessary information about withdrawal period of drugs.

Seventy two samples (33.8%) of the tissue samples were positive to the antibiotic residues, when using whole tissue test with mean inhibition zone equal to 4.5mm. (67.6%) of liver tissues were positive with mean inhibition zone of 5.1 mm , 9 (12.7%) and 15 (21.1%) samples were positive in muscle and gizzard tissues with mean inhibition zone equal to 3.5 and 5 mm respectively . The inhibition zone of more than two mm is considered as the standard positive diameter for calculating the positive inhibition zone. (Table 1).

**Table 1:** The comparison between the whole tissue and disc assay methods

| Organ | Whole tissue method | | Disc assay method | | |
|---|---|---|---|---|---|
| | % +ve | Diameter mm-mean inhibition zone | % +ve | Diameter mm mean inhibition zone | Level of Significance |
| Liver | 48 (67.6%) | 5.1mm | 41 57.7%) | 4.4mm | 0.38 |
| Muscles | 9 (12.7%) | 3.5mm | 3 (4.7%) | 5.5mm | 0.009 |
| Gizzard | 15 (21.1%) | 5mm | 20 (28%) | 5.1mm | 0.01 |
| Total | 72 (33.8%) | 4.5mm | 64 (30.0%) | 5mm | |

In the disc assay method, 64 (30%) of samples were positive with mean inhibition zone equals to 5mm, 41(57.7%) of the liver samples were positive with mean inhibition zone equals to 4 mm.,3 (4%) and 20 (28%) of muscle and gizzard samples were positive when using disc assay method with mean inhibition zone of 5.5 mm and 5.1 mm respectively (Table 1).

The difference between the inhibition zones diameter in the different tissues was compared statistically using Analysis of Variance test. (ANOVA) .The difference between the mean inhibition zone in the Gizzard using disc assay method and whole tissue method was found to be statistically significant, p (0.017). The difference between the mean inhibition zone in the poultry muscle using disc assay method and whole tissue method was found to be statistically significant,p (0.030). The difference between the mean inhibition zone in the poultry Liver samples using disc assay method and whole tissue method was found to be statistically insignificant, p (0.88). (Table 2).

**Table 2.** ANOVA test results

| Tissue | DF | Mean Square | F | Sig |
|---|---|---|---|---|
| Gizzard | 1 | 24.379 | 5.936 | 0.017 |
| Muscle | 1 | 8.092 | 4.920 | 0.03 |
| Liver | .1 | 836 | .836 | .022 |

Using t- test to compare the diameter of inhibition zones between the total results of different tissues, it was found that there was significant difference p (0.01) between muscles diameter inhibition zone and the diameter of inhibition zone of gizzard, also the difference is significant (0.00) between the diameter of inhibition zone in liver and gizzard and between liver and muscles (0.00).

**Table 3** t- Test for comparison between different tissues

| | | Paired Differences | | | | | T | Df | Sig. (2-tailed) |
|---|---|---|---|---|---|---|---|---|---|
| | | Mean | Std. Deviation | Std. Error Mean | 95% Confidence Interval of the Difference | | | | |
| | | | | | Lower | Upper | | | |
| Pair 1 | muscle–gizzard | -.66197- | 2.31729 | .27501 | -1.21046- | -.11348- | -2.407- | 70 | .019 |
| Pair 2 | liver–gizzard | 1.91549 | 2.80686 | .33311 | 1.25112 | 2.57987 | 5.750 | 70 | .000 |
| Pair 3 | liver–muscle | 2.57746 | 2.53919 | .30135 | 1.97645 | 3.17848 | 8.553 | 70 | .000 |

**Table 4:** One-Sample t- Test

| | Test Value = 3 | | | | | |
|---|---|---|---|---|---|---|
| | T | Df | Sig. (2-tailed) | Mean Difference | 95% Confidence Interval of the Difference | |
| | | | | | Lower | Upper |
| Liversize | .096 | 70 | ..000 | .02817 | -.5543- | .6107 |
| Gizzardsize | -7.290- | 70 | .000 | -1.88732- | -2.4037- | -1.3710- |
| Musclesize | -16.299- | 70 | .923 | -2.54930- | -2.8612- | -2.2374- |

The inhibition zone of three mm is considered as the standard positive diameter for the inhibition zone , there was no significant difference between this standard and the mean inhibition diameter obtained by muscle with $\alpha \leq .05$, but the diameter of the mean inhibition zone vary significantly between the standard and the mean diameter of inhibition zone in the gizzard and the liver, with $p \leq (0.00)$.

**Table 5** Disc assay * whole tissue methods Cross tabulation

| Count | | Whole | | Total |
|---|---|---|---|---|
| | | positive | negative | |
| Disc | positive | 64 | 10 | 74 |
| | negatve | 35 | 104 | 139 |
| Total | | 99 | 114 | 213 |
| Measure of Agreement | | Kappa | .568** | |
| Pearson Chi-Square | | 72.963[a] | .000** | |

** = Highly significant
Percentage agreement = 78.8%

As shown in table 5 there was a highly significant association between the disc assay method and the whole tissue tests method. The measure of agreement between the two tests method was highly significant using Kappa statistic 0.568 and the percentage agreement was 78.8%. (Table 5)

## DISCUSSION

Now a days the presence of antimicrobial residues in the different animal origin food and products is becoming a matter of concern for public health. To protect the health of consumers, maximum residue limits (MRLs) of pharmacologically active substances in foodstuffs of animal origin have been established by the (EU) No. 37/2010 Commission Regulation (European Commission, 2010.). Reports of residue tests should not mention positive and negative results, but instead the terms 'non-compliant' and 'compliant' should be used. (Anon 2002) Vast number of studies were being carried out to investigate the presence of the different veterinary drugs including antibiotic residues in animal products due to the side effects they impose on the health of consumers. Okerman, *et al* 2007 reported that *Bacillus subtilis* was one of the best bacteria can be used for the detection of the antibiotic residues.

Poultry farmers use different antibiotics for different purposes. In this study most of the farmers used tetracycline alone (27.5%) or in combination with other drugs (62.5%) . Although all the poultry farms had an assigned veterinarian but the administration of antibiotics was followed by the farmers in (50%) of the farms. Antibiotics were used for treatment of diseases of respiratory signs in most (75%) of the farms, and most of the farmers (75%) lack the necessary information about the withdrawal period of drugs. A previous study carried in Khartoum- State showed that tetracycline also in addition to penicillin were used extensively in poultry farms either for prevention (50 % of visited farms) or for treatment (37.5%) (Adil M Salman *et al.*, 2012). A study carried out in Nigeria showed that all investigated farms used a drug at least once, nine used antibacterial drugs for either prophylaxis, therapy or both while none of the farms observed drug withdrawal period( Kabira *et al.*, 2004).

Different techniques are used for detection of antibiotic residues in poultry meat and tissues ranging from simple methods such as microbiological methods up to using liquid chromatography–mass spectrometry ( Adla and Nada 2019). In the microbiological methods, Okerman *et al.*, (2007) reported that *Bacillus subtilis* was one of the best bacteria that can be used for the detection of the antibiotic residues. Also either the whole tissue under study or the disc paper moist with tissue fluid techniques are commonly used (Okerman *et al.*, 2004).

This study revealed that the whole tissue method detected higher percentage of positive samples (33.8%) compared to disc assay method (30%). Using chi square test there was highly significant association between the two test methods, the Kappa statistic for the agreement between the two methods was statistically significant 0.568 with ($P \leq 0.05$) and the

percentage agreement between the two tests was 78.8% , which indicates that both test methods had almost equal reliability . Okerman *et al.,* (2004) , reported that analysing undiluted meat fluid instead of whole tissue is not an advantage, as the detection capability is not enhanced and the sample preparation takes more time.

The mean higher diameters of residue inhibition zone in all tissue samples (5mm) was detected by disc assay method. The highest number of positive samples was detected in the liver samples in both methods 48 (67.6%) and 41 (57.7%) in whole tissue and disc assay methods, respectively. The mean highest (5.5 mm) and lowest (3.5mm) diameter of residues inhibition zone was detected in muscle samples using disc assay and whole tissue methods, respectively. But the mean inhibition was the highest in gizzard using both tests 5 mm in the whole tissue method and 5.1 mm in the disc assay method. Using filter paper method or the disc assay method was found to have no significant effect on the frequency of antibiotic residues detection when the used tissue was the liver, but when the used tissue was the muscle or gizzard the two tests differed significantly.

Among the vast number of similar previous studies the liver was the highest antibiotics contaminated organ, Chicken liver contained the highest of proportion of antibiotic residues than the rest of three samples. The current study showed that the highest frequencies of antibiotic residue was in liver (48, 67.6%). This result was in line with Sarmina Sattar *et al.,* (2014) who showed higher antibiotic residues also in liver; these included tetracycline (48%) followed by ciprofloxacin (44%), amoxicillin (42%) and enrofloxacin (40%). Hind A.Elnasri *et al.,* (2014) detected high positive samples in muscle and liver (29.2%, 28.3%), respectively.

This finding was similar to that reported by Naeem *et al.,* (2006) and Shabeer Ahmad (2016) who reported that the percentages of positive samples in liver tissues were higher for antibiotic residues detection compared to kidneys. Muhammad Akbar Shahid, *et al* (2007) comparing the antibiotic residues in different poultry tissue liver detected a higher positive antibiotic residues (39.4%), compared to the percentage detected in this study. In studies in liver of chickens (Hind, A. Elnasri *et al.,* 2014) detected 28.3% positive samples which was lower to what was detected in the current study 44(62%).

Other studies also showed the presence of antibiotic residues in poultry meat, 45.7% of samples were positive for quinolone (Hakem *et al.,* 2013), which was higher compared to the present study, but in Belgium, Okerman *et al.,* (2004) reported almost similar results in poultry muscle (11.3%) and (8.33%) in thigh and breast muscles, respectively but site of muscle chosen for antibiotic residue detection had no significant effect . Reyes *et al.,* (2008) also detected no significant difference of antibiotics residues between the different sections of breast tissues and thigh tissues. Muhammad Akbar Shahid, *et al.,* (2007) showed that 20.6% of poultry muscle samples were positive for the antibiotic residues which was higher than the percentage detected in this study. Another investigation carried out in different areas of the Dominican Republic showed that poultry meat contained quinolone residues with different concentrations (Silfrany *et al.,* 2013). In Egypt, it was reported that 34% of tested broiler fillets were positive for Oxytetracycline (Mohamed *et al.,.,* 2013).

Despite of the lack of researches on the antibiotic residue in the gizzard, this study revealed appreciable accumulation of antibiotic in this organ.

The standard antibiotics inhibition zone was reported by Mariel G. (2009) to be three mm or more when this compared with liver inhibition zone detected in this study, there was no statistical significant difference between the standard and the inhibition zone produced by the liver tissues, in contrast to the muscles and gizzard were found to be statistically significant ($P \leq 0.05$).

As a consequence, there is a strong possibility that antibiotic residues may appear in poultry products, this may be attributed to the carelessness about veterinary drugs withdrawal period as the main risk factor leading to drug residues in chicken's meat. Adil M. Salman *et al.,* (2012) reported that 43.3% of the veterinarians at veterinary pharmacies practiced whole sale of antibiotics to the farmers, 60%guided them to restrict dose and only, 37.3% advised the owners about the routes of administration. However, only 56.7% from all interviewed, veterinarians advice the farms' owners about the withdrawal period (Fathalrhman *et al.,* 2016). In this study the vast majority of farmers s .do not a ware of the drug withdrawal period .The drug group is also another factor that must be taken into account due to variation of using frequencies. Hakem *et al.,* (2013) observed that most of positive samples cases were found contaminated by β-lactams and/or tetracyclines (75.81%).

## CONCLUSION

The higher results obtained under the present study may be attributed to the extensive use of antibiotics by farm owners, widespread misuses of antibiotic in poultry farms and the lack of awareness of farmers regarding the recommended withdrawal periods of drugs. Proper governmental regulations, monitoring and awareness related to the poultry raising and meat consumption among the public is warranted in the future.

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