RISK FACTORS AND REFERENCE VALUES OF ANTIBIOTIC RESIDUES IN IMPORTED RED MEAT AND THEIR IMPACTS ON GENOTOXICITY AND DNA DAMAGE IN HUMAN LYMPHOCYTES

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ABSTRACT

Antibiotic residues in food are accelerate the development of antibiotic resistance in bacteria, transfer of antibiotic-resistant bacteria to humans, cause allergies (penicillin), and cancers. This study was designed to investigate the risk factors of some antibiotic residue in imported red meat at Baghdad markets, their reference values, and their impact on genotoxicity and DNA damage in human cells.Atotalof144 meat samples were collected from January to July 2022.The results showed that the overall contamination percentage was35.4%. Buffalo meat showed the lowest contamination percentage 15% whereas the minced beef showed the highest (94.5%) (OR=402.33).Tetracycline residues detected in 42.8% of samples with significant risk (P=0.0001)(OR=33.37;95%CI=7.74-143.94).The mean of tetracycline was 83.80 ppb with lower and upper limits of 56.7, 110.9 ppb, respectively. Blood samples of 18 healthy males were used to isolate lymphocytes to detect the genotoxicity and DNA damage of antibiotic residues. Results showed significant effects of antibiotic residues on genotoxicity and human DNA damage. These results also showed that although the antibiotics residues concentrations are below the allowed maximum residues limit, this does not reduce the hazards of these residues on public health resulting from their accumulative increase in consumers' tissues.

Keywords: antibiotic residues, referencevalues, genotoxicity, red meat, Iraq, food safety

عوامل الخطر والقيم المرجعية لبقايا المضادات الحيوية في اللحوم الحمراء المستوردة وتأثيراتها على السمية الجينية وتلف الحمض النووي في خلايا الإنسان

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المستخلص

تساهم بقايا المضادات الحيوية في الأغذية في تسريع تطور مقاومة المضادات الحيوية في البكتيريا، ونقل البكتيريا المقاومة للمضادات الحيوية إلى البشر، وتسبب الحساسية (البنسلين)، والسرطانات.صُممت هذه الدراسة للتحري عن عوامل الخطر لبعض بقايا المضادات الحيوية في اللحوم الحمراء المستوردة في أسواق بغداد، وقيمها المرجعية، وتأثيراتها على السمية الجينية وتلف الحمض النووي في الخلايا البشرية. تم جمع 144 عينة لحوم من كانون الثاني إلى تموز 2022. أظهرت النتائج أن نسبة التلوث الكلية كانت 35.4٪. أظهر لحم الجاموس أقل نسبة تلوث 15٪ بينما أظهر لحم البقر المفروم أعلى نسبة (94.5٪)(OR=402.33) تم الكشف عن بقايا مضاد التتراسيكلين في42.8٪ من العينات مع خطر معنويOR=(P=0.0001)(33.37؛95٪ CI 143.94– 7.74)=وبلغ متوسط تركيز التتراسيكلين 83.80 جزء في المليار مع حد أدنى وأعلى 56.7, 110.9 جزء في المليار على التوالي. وتم استخدام عينات دم 18 ذكر سليم لعزل الخلايا الليمفاوية للكشف عن السمية الجينية وتلف الحمض النووي لبقايا المضادات الحيوية. وأظهرت النتائج تأثيرات كبيرة لبقايا المضادات الحيوية على السمية الجينية وتلف الحمض النووي البشري. وأظهرت هذه النتائج أيضًا انه على الرغم من أن تركيزات بقايا المضادات الحيوية أقل من الحد الأقصى المسموح به للبقايا ،إلا أن هذا لا يقلل من مخاطر هذه البقايا على الصحة العامة الناتجة عن زيادتها التراكمية في أنسجة المستهلكين

الكلمات المفتاحية :بقايا المضادات الحيوية، القيم المرجعية ،السمية الجينية، اللحوم الحمراء، العراق، سلامة الغذاء

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INTRODUCTION

Antimicrobials are widely used to control, prevent, and treat infection, and to enhance animal growth and feed efficiency (33). As a result of the extensive use ,the antibiotics residues are often found in animal products, such as meat, milk, and eggs that eventually find humans to be the ultimate consumers of these antibiotic residues (39, 38). Red meat and meat products are considered good foods with high nutritional value because they contain vitamins, proteins, fats, and minerals. The available local red meat is not enough to meet consumer demand. (1,3, 6, 28). Consuming foods including red meat that contaminated with antibiotic residues could increase bacterial resistance to antibiotics, posing a significant public health risk (9, 43, 24).These antibiotic-resistant bacteria were transferred to the human and can cause severe and often fatal infectious diseases (16). Ramatla *et al*., (44) detected different concentrations of antibiotic residues using HPLC that were in the ranges of 20.7–82.1,41.8– 320.8, 65.2–952.2, and 32.8–95.6 μg/kg for sulphanilamide, tetracycline, streptomycin, and ciprofloxacin, respectively. In Iraq, Ahmed *et al*.,(7) found the antibiotic residues in blood of sheep (0.413 ppb), and heifers (0.358ppb). Antibiotic residues as genotoxic agents could cause DNA damage in cells, leading to mutations and cancer (14).Assessing the genotoxic potential of food contaminants including meat is crucial for understanding their impact and risk on human health (19, 13, 36). Micronucleus (MN) tests are used to detect chromosome fragments in cells to demonstrate potential genetic damage (45). Chromosomal Aberration tests identify structural changes in chromosomes, revealing mutagenic effects of antibiotic residues in foodstuffs (29). DNA damage can be detected by comet assay through gel electrophoresis (18).These tests were used to detect the gene toxic effects and DNA damage induced by antibiotic residues in red meat in the Baghdad markets. This research was conducted to cover the great gap in Iraqi studies that investigate the effects of antibiotic residues on genotoxicity and DNA damage in human lymphocytes.

MATERIALS AND METHODS

In this study, a total of 144 imported red meat samples were collected from various markets across Baghdad (Al-Karkh and Al-Rusafa districts). The sample collection spanned from JanuarytoJuly2022 and included beef, cows, buffalos, veal, and minced beef.

Preparation of meat samples

The homogenized sample (10g) was transferred to a shaking bottle ,a solution of a cetonitrile containing 1% formic acid (50 mL) was added, and the resulting mixture was shaken for 30 min. After this time, anhydrous magnesium sulphate (4 g) and sodium chloride (1 g, to increase the ionic strength and distribution efficiency) were added, and the mixture was shaken for 10 min prior to centrifugation for 10 min at 4000 rpm. The supernatant (25 mL) was then added to acetone containing 2% diethylene glycol (0.2 mL) and the solvent was evaporated to dryness. The resulting extract was re dissolved in a mixture ofacetone/hexane (2:8, v/v, 4 ml), which was subsequently loaded onto an SPE-florisil cartridge that was activated with hexane(5 ml) and acetone/hexane (2:8, v/v,5 ml) for sample purification.

Liquid Chromatography - Mass Spectrometry (LC/MS).: The ultra-high-performance liquid chromatographic system by Liquid chromatography- tandem mass spectrometry LC-MS/MS AGILENT 1290 USA coupled with the QTRAP®6500+ MS system from AB Sciex (Framingham, MA, USA)was used as standards method for analysis of contamination with antibiotic residues. Blood samples collected from 18 healthy individuals were used to isolate lymphocytes for conducting genotoxicity test (49)

Genotoxicity effects and DNA damage analysis

1- The Micronucleus (MN) test was applied according to Fenech (22), Al-Sudany (11) and Ad'hiah *et al*., (5) to estimate the MN formation. Briefly, after cell preparation and addition of cytochalastin B, they were fixed and smeared on clean slides followed by staining with Giemsa. The slides were examined under oil immersion lens (100X), and inspected for the formation of micronucleus. The micronucleus index wasscoredusing1000cellsbyusing the following equation:

Micronucleus index (micronucleus/cell) =

$$\left(\frac{\text{Number of Micronuclei}}{\text{Total Count of Cells}}\right) x\,100$$

2- The Mitotic Index (MI) and chromosomal Aberration (CA) were estimated according to Shubber (49).In brief, the estimation of the MI and CA in human blood, cells were prepared, PHA stimulated and olchirin addition and then harvested to be fixed and stained by Giemsa, then examined microscopically. The percentage rate for only the divided cells was then determined using the formula below:

Mitotic index=

$$\left(\frac{\text{Number of the divided cells}}{\text{Total number of the cells}}\right) x\,100$$

3- DNA damage can be detected by comet assays after mounting cells, microscope slides in a thin layer of agarose gel, the cells were lysed to eliminate all cellular proteins. The DNA was unwound, electrophoresed, and then fluorescently dyed after that relaxed chromatin or fragmented DNA fragments (damaged DNA) migrate away from the nucleus during electrophoresis. The amount of DNA released from the comet's head directly relates to the amount of DNA damage.(10).

Statistical analysis

Data were analyzed using SAS program (version 9.1) (46). Independent t test was used to assess the significant difference between two means and Chi-square test was used for proportions. If any expected frequency is less than 5 in more than 20% of cells, the Chi- square tests is inappropriate, therefore, it is usually considered acceptable if Yates' correction is employed. Odds ratio were estimated using MedCalc program (35).Reference values were estimated by using reference value advisor (25).$P<0.05$ is considered significant.

RESULTS AND DISCUSION

In the current study, the Liquid Chromatography-Mass Spectrometry (LC/MS) was utilized to quantitative and qualitative detect the presence of antibiotics in various meat samples. This qualitative analysis provided valuable insights into the nature and types of antibiotic residues that present in meat products which may assist in the assessment of food safety and compliance with regulatory standards. Table(1) provides a comprehensive over view of the results to determine the contamination of different meat types with antibiotics residue. The antibiotics residue contamination contributed to 35.4% of all meat types. The minced meat had the highest percentage of antibiotics residue 94.5% (OR=402.33;95% CI=39.4-4111.5) and the cow meat had 66.6% of contamination (OR=11.33;95%CI=2.04-63.08) followed by veal meat of 24.4% (OR=136.8; 95%CI=28.3-653.5) and the lowest contaminated meat was detected in buffalo meat with a percentage of15%.The high percentage of contamination of minced meat could be attributed to the processing practices of minced meat, which often involves mixing meat from multiple sources and handling and mixing processes (31).This ratio of contamination in cow meat was more than that observed in buffalo meat suggests that cows may be subjected to more intensive antibiotic treatments and difference in life span as well as rearing circumstance compared to other livestock (30, 37).

Table1.Percentages and health risk of contamination of meat with antibiotics residue in Baghdad meat market

Type of meat	Total No.	Antibiotics No.(%)	Yates Chi-Square	Yates P-value	Odds Ratio (95%CI)	P-value
Buffalo	**20**	**3(15%)**	**36.01**	**<0.0001**	**Reference**	
Cow	**12**	**8(66.6%)**			**11.33(2.04-63.08)**	**0.005**
Veal	**94**	**23(24.4%)**			**136.8(28.3-653.5)**	**<0.0001**
Minced	**18**	**17(94.4)**			**402.33(39.4-4111.5)**	**<0.0001**
Total	**144**	**51(35.4%)**				

Table (2) shows the contamination rates for various types of antibiotics residue. In minced meat samples, it was noted that the highest contamination rate with Tetracycline residue (42.8%) with significant risk (P=0.0001)OR=33.37; 95%CI=7.74-143.94, followed by bi- combination contamination 28.6% (OR=17.80; 95%CI=4.09-77.68; P<0.0001), and tri- combination 19.6% (OR=10.22; 95%CI=2.28-45.69; P=0.002), then macrolides at a non- significant rate of 8.8% (OR=3.31; 95%CI=0.67-16.38; P=0.14) and the lowest was sulphonamides at 2.2%. The significant risk of abundant tetracycline residue in red meat could be explained by the over use of this antibiotic in disease prevention and treatments (47). Many studies reported that the tetracycline levels were above the maximum residual limit (8, 20, 12).

Table 2. Percentage and the risk factors associated with different types of antibiotics residue present in red meat contamination

Type of antibiotics	Contamination NO. (%)	Chi-square	P-value	Odds Ratio	P-value
Sulphonamides	2(2.2%)	48.50	<0.0001	Reference=1	-
Macrolides	8(8.8%)			3.31(0.67-16.38)	0.14NS
Tetracycline	39(42.8%)			33.37(7.74-143.94)	<0.0001
Bi-combination Tetracycline +Penicillin Tetracycline +Sulphonamide Tetracycline +Macrolides Aminoglycoside+Macrolides Sulphonamide+Aminoglycoside	26(28.6%)			17.80(4.09-77.68)	0.0001
Tri-combination Tetracycline+Penicillin+Macr olides Sulphonamide+Macrolides+ Aminoglycoside	18 (19.6%)			10.22(2.28-45.69)	0.002
Total	93 (64.6%)				

The reference values of the Tetracycline (sample number=63) and penicillin (sample number=24) were determined using reference value advisor. Results showed that Tetracycline had a lower limit of 56.7 ppb and an upper limit of 110.9 ppb with normal distribution (Figure 1), and LC/MS profile of Tetracycline was shown in Figure (2). The lower limit of penicillin was 10.62 ppb and the upper limit was 11.48 ppb (Table 3) with also normal distribution (Figure 3) and LC/MS profile of penicillin was shown in Figure (4). In general, these values are considered below the acceptable maximum residue limit (MRL) of codex, whichare100 and 50 ppb for tetracycline and penicillin, respectively.

Table3.Reference values of tetracycline and Penicillin using reference value advisor

Type of antibiotic	No	Mean± STD ppb	Lower limit(LL)	Upper limit(UL)	90%LL	90%UL	Method (ppb)
Tetracycline	63	83.80±13.5	56.7	110.9	52.5-61.0	106.3-115.2	Codex 100
Penicillin	25	11.05±0.21	10.62	11.48	10.51-10.73	11.36-11.60	Codex 50

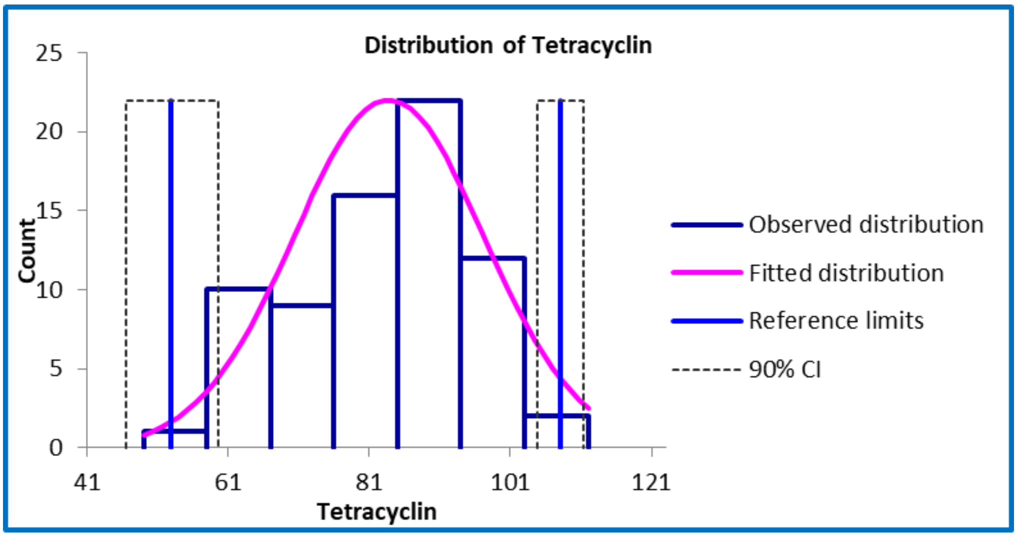

Figure1.The distribution of tetracycline (normal distribution)

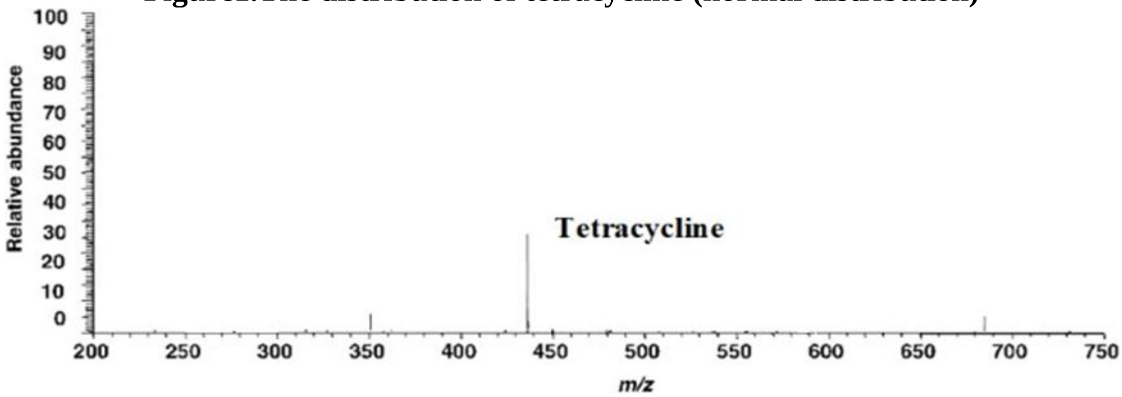

Figure2.LC–MS/analysis of Tetracycline residue in red meat.

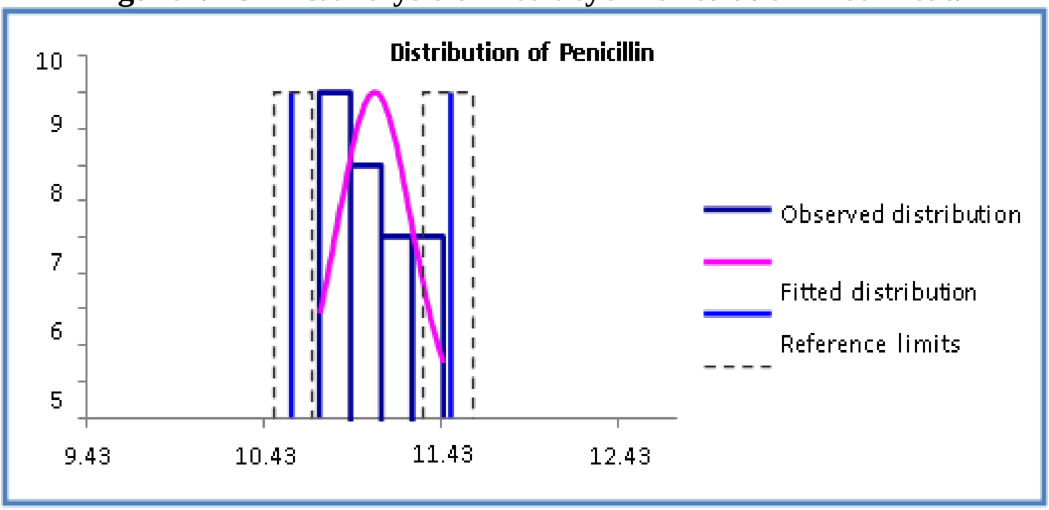

Figure3.The distribution of penicillin (normal distribution)

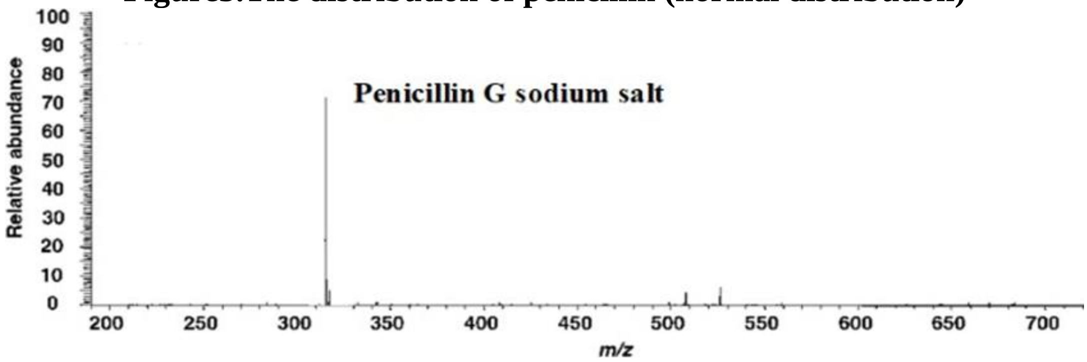

Figure4. LC–MS analysis of Penicillin residue in red meat

Overall, our study contributes to the ongoing discourse on food safety and agrees with the urgent call for comprehensive surveillance and strict enforcement of antibiotic use in live stock to ensure food safety and public health security. These efforts are crucial to mitigate the risks that are associated with antibiotics residue and to prevent the escalation of antibiotic resistance globally. In a study carried out by Er *et al*.,(20) in Turkey, the quinolone antibiotics residues were detected in 57.7% of beef meat in Ankara. Moreover, the misuse of antibiotics as a tool of promoting livestock production lead to deposition in red meat then cause a serious food safety concern due to their potential adverse health effects on consumers (12).Antibiotic residues can induce allergic reactions, promote the development and spread of antibiotic-resistant bacteria, and even cause severe health issues such as cancer, anaphylactic shock, reproductive disorders and chronic toxic effects on human health, such as liver or kidney damage(32, 26).The interaction between drug-drug could be another contributing factor that influencing the pharmacokinetics and clinical effects of drugs metabolism via either induction and/or inhibition of cytochrome P450 (CYP450) (34). It is interesting to know that antibiotics residue can remain in the animal's tissues and end up in the meat that is consumed by humans (44,42).In Iraq, a recent report by Wali and Deri (52) found that contamination rate of Tetracycline was18.12% while Sulphonamide was 11.87% in beef meat, minced meat, and kebab. It has been found that 22.2% of examined sheep meats had antibiotics in Erbil, north of Iraq, which is considered a high.

Impact of Antibiotics Residue in meat on some Genotoxicity and human DNA damage: The genotoxic effects of antibiotics residue in red meat were examined using three different genotoxic markers :the chromosomal aberrations (CA), micronuclei (MN), and micronucleus incidence (MI) (Table 4). Results revealed that the MN ratio had a significant increase in antibiotics-contaminated samples (0.007±0.0005a) indicating a high potential for genotoxic risk. Specifically, samples contaminated with antibiotics residue showed an increase in CA from0.14±0.02 of the free-contaminated samples up to 0.20±0.02 of those contaminated with antibiotics residue. They also had an increase in the number of MN from 4.74±0.29 to 8.19±0.53 indicating significant changes in genetoxic markers and confirming a pronounced genotoxic health risk. The difference in MI between two groups was not significant. The presence of antibiotics residue in red meat and the associated genotoxic effects can have significant public health implications, such as mutagenesis and carcinogenesis (23),antibiotic resistance (17),reproductive and developmental toxicity (2, 27), or auto immune disorders (4).In addition, antibiotic residues, such as tetracycline's and sulphonamides, can be mediated through DNA strand breaks and oxidative DNA damage in mammalian cells (51, 36).Furthermore, antibiotic residues can increase DNA damage markers, such as micronuclei formation and comet assay parameters, in various animal tissues (26, 48)**.** Some antibiotics can also impair DNA repair mechanisms, allowing DNA damage to accumulate in cells (21). To alleviate the risks associated with antibiotics residue in red meat, strict regulations, monitoring programs, and proper withdrawal periods for treated animals are crucial. Additionally ,promoting responsible antibiotic use in live stock production and exploring alternative strategies for disease prevention and control can help reduce the presence of antibiotics residue in the food supply (50).

Table 4.The effect of antibiotics residue on genotoxic effect on micronuclei (MN), chromosomal aberrations and MI in human cells

Status	No	CA	MI	No of cells Contain MN	No. of MN	Ratio MN/1000
Contaminated-free	26	0.14±0.02	9.98±0.05	4.15±0.31b	4.74±0.29b	0.004±0.0003b
Antibiotics	51	0.20±0.02	9.92±0.04	5.68±0.29a	8.19±0.53a	0.007±0.0005a

P-value	144	0.06	0.36	0.001	0.0001	0.01

The chromosomal aberrations which lead to genotoxicity were also observed in meat samples which was detected as gaps in chromosome and chromatid (Figure 3), representing breaks affecting the entire chromosome, but not completely separating any part.

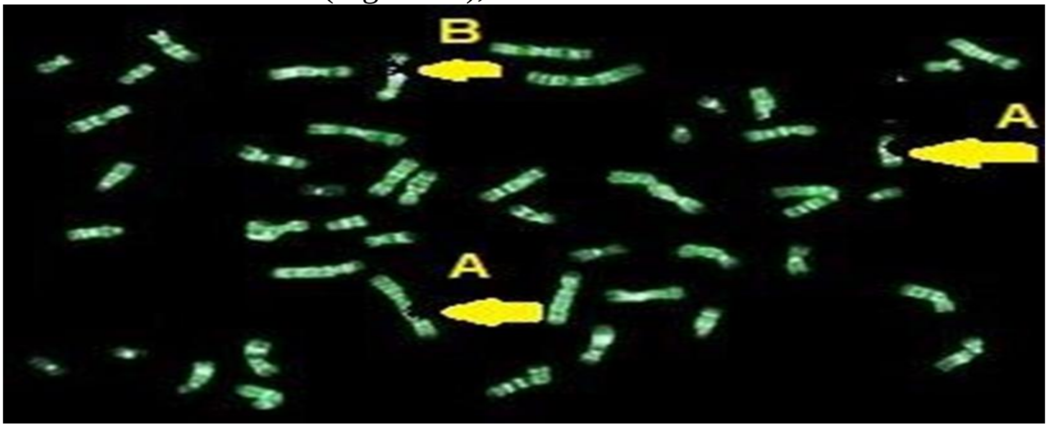

Figure 3. Chromosomal aberrations in red meat(100X).A.chromatid gaps (G'). B. chromosome gaps (G'') affecting whole chromosomes. Marked as "A"are chromatid gaps (G'), which are clear breaks within a single chromatid ofa chromosome. Marked as "B" are chromosome gaps (G'').

The micronuclei results (Figure 4) showed that each cell has at least one micronucleus. The presence of this micronucleus indicates that the cell has undergone some form of chromosomal breakage or malfunction during cell division.

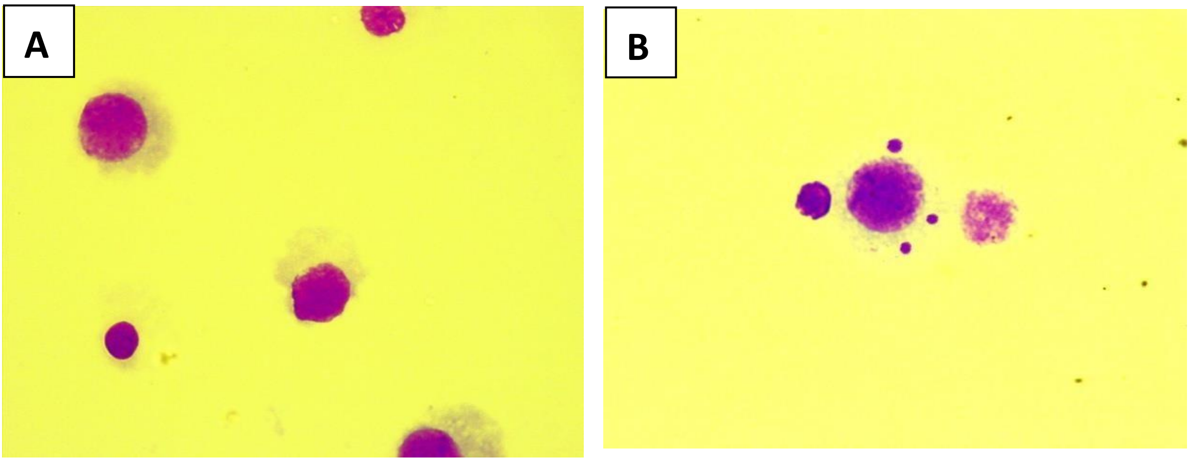

Figure 4. Micronuclei appearance in cells exposed to genotoxic agents (antibiotics). A. normal cells. B. cells with multiple micronuclei

Impact of Antibiotics Residue in red meat on DNA damage in human cells

Results of antibiotics residue contamination indicate a higher DNA damage in human lymphocytes;25.27±3.84% in the head and 27.56±3.25% in the tail (Table 5).These values are higher compared to 20.21±2.22 % and 16.24±2.65 % in free contaminated samples, respectively.

Table5.The effect of antibiotics on DNA damage in human by using comet assay

Status	Sample No	%DNA damage in head	%DNA damage in tail
Contamination. free	26	20.21±1.22b	16.24±2.65b
Antibiotics	51	25.27±1.24a	27.56±3.25a
P-value		0.01	0.02

Figure(5),shows the difference between normal and abnormal cells for DNA damage in head and tail, where normal cells are round and uniform in appearance with evenly distributed fluorescence suggesting undamaged cells (Figure 5A),whereas those contaminated with antibiotics residue showed abnormality with irregularly shape and vary in size, some showing intense fluorescence and others more diffuse fluorescence, suggesting DNA damage (Figure 5B).

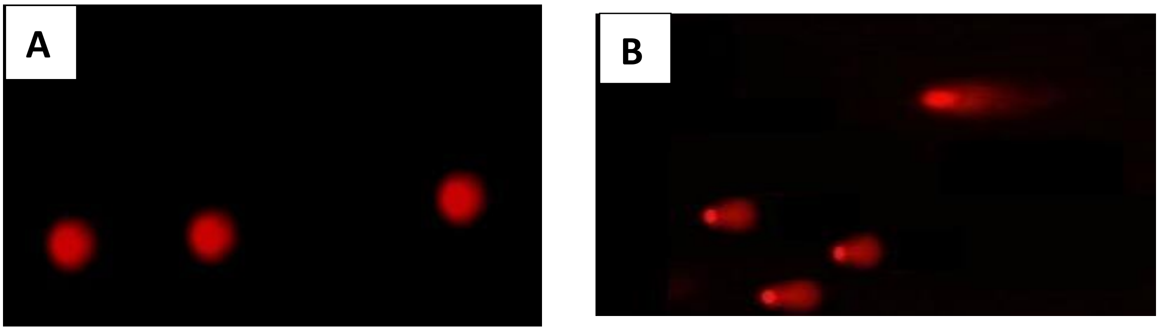

Figure 5.A.Control group showed no DNA damage in control group cells. B. Antibiotics residue contaminated meat samples with a significant migration of DNA from head to tail

The results concerning genotoxic effects and DNA damage agrees with several studies (41, 32, 15). The mechanism of DNA damage due to antibiotic residues in red meat could be caused by oxidative stress generating reactive oxygen species (ROS) which can directly damage DNA by causing strand breaks, base modifications, and DNA-protein cross-linking (44)**.**Certain antibiotics or their metabolites can form DNA-reactive intermediates that covalently bind to DNA, causing adducts and mutations (40), Additionally, antibiotics like fluoroquinolones can inhibit topoisomerase enzymes, the crucial for DNA unwinding and replication leading to DNA double-strand breaks and chromosomal aberrations(53)**.**Finally, some antibiotics can impair DNArepair mechanisms, allowing DNA damage to accumulate in cells (21).Therefore**,** the consistency of our results with these studies indicates the genotoxic potential of antibiotics residue in meat, aligning with broader concerns about antibiotic using in agriculture, meat production and its risk implications on human health. Although global studies are interested in studying the impact of antibiotics residue on public health, there has been a significant gap in local researches. Our study fills this critical void by providing an initial insights into how antibiotics residues in consumed meat causes genotoxic affect and DNA damage in humans. In addition, Tetracycline and bi-combination antibiotics residues were significantly present in food samples with 42.8% and28.6%, respectively.

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