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**Abstract:**
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Inspection of Antimicrobial Remains in Bovine Milk in Egypt and Saudi Arabia Employing a bacteriological test kit and HPLC-MS/MS with Estimation of Risk to Human Health

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Abstract

Veterinary medicine uses antibiotics randomly for treatment and growth promotion. Milk of dairy animals contains substantial quantities of antibiotics that have harmful effects on health. It is therefore necessary to test commercially available milk with immunological, chromatographic, or microbiological methods to avoid antibiotic residues. This study aims to perform a microbiological test, followed by a quantitative confirmation analysis, on raw milk to assess whether antibiotic residues are present. Tests were conducted on 200 milk samples taken from markets and farms in Saudi Arabia and Egypt. The microbial inhibitor test (Delvotest SP-NT) revealed that 40 samples were positive. The positive samples were tested using liquid chromatography-tandem mass spectrometry (LC-MS/MS) as a confirmatory quantitative test for 29 antibiotics that belong to five groups: tetracyclines, sulfonamides, fluoroquinolones, macrolides, and lactamases. Only four samples tested positive for oxytetracycline residues above the maximum residue limit. Based on these results, researchers suggest a monitoring system that takes into account both microbial and HPLC-MS/MS methods when detecting antibiotic residues in Bovine milk. The analysis of risk to human health revealed that antibiotic remains in found concentrations do not pose any health risks to consumers.

Keywords: Screening; Bovine Milk; Antimicrobial Residues; Oxytetracycline, Bacteriology; HPLC-MS/MS.
1. Introduction

Antibiotics are a broad group of medicines that are utilized to destroy or prevent bacterial microorganisms from developing. In the field of human medicine, they are used to treat a variety of microbial infections. In the field of animal husbandry, they are widely used for infection treatment, but they are also illegally used for prophylaxis and growth promotion, which can boost a farm's financial return [1,2]. Utilizing these products may deliver residues into milk, triggering unfavorable allergic reactions in humans [3]. Moreover, antimicrobials may cause antibiotic-resistant bacteria to flourish, which can cause serious medical conditions [4]. Various governments have established monitoring projects to establish antibiotic concentrations in food and a maximum residue limit (MRL) for them [5,6]. Consequently, the European Union (EU) has prohibited the illegal growth promotion usage of antibiotics. Global organizations and State governing agencies like the World Health Organization (WHO) and the United Nations Food and Agriculture Organization (FAO) (collectively formed the Codex Alimentarius), and the United States Department of Agriculture (USDA) have established MRLs for medicines intended in veterinary usage and their presence is allowed in nutrients of animal source [7]. In developing countries, however, veterinary drug abuse is detected at shocking rates due to inadequate supervision and limited analytical controls [8].

Checking nutrients of animal sources for the existence of antibiotic residues is generally executed by screening procedures, which comprise microbiological tests, and confirmatory quantitative procedures including liquid chromatography coupled to mass spectrometry [9]. Microbiological tests contain two major groups: tube and multi-plate tests. The multi-plate test consists of dishes holding agar medium with diverse indicator bacteria. Specimens are placed on the top of the agar surface, then, after incubation, the growth of bacteria will change the opacity of
the agar. If antibiotic residues are contained in the specimen, inhibition of the growth of bacteria will occur, producing a clear zone nearby the specimen. A disadvantage of this test is that it is time-consuming due to the continuous need for fresh agar and fresh bacterial cultures; hence, they are inappropriate for macroscale [10].

Tube tests, which are commercially accessible, employed for onsite screening of antibiotic residues. They consist of ready-to-use tubes comprising microorganisms on focus together with the nutrients, indicator, and an agar medium [11]. The tube methods are used efficiently for the examination of residual antibiotic drugs in the livestock food than the multi-plate as it is neither time-consuming nor exhausting [12]. The most common indicator bacterium used in these inhibition tests is *Geobacillus stearothermophilus*, because of several reasons such as low contamination level, tolerance to high temperature (55°C), and shorter incubation time (less than 4 h) compared to other bacteria. Furthermore, it is more susceptible to antibiotics, especially, β-lactams [13].

For example, the Delvotest® SP NT (produced by DSM Food Specialties Ltd., The Netherlands), which is a standard diffusion test for the screening of antibacterial substances in dairy milk through inhibiting the growth of *Bacillus stearothermophilus* strain, that is susceptible to several antibiotics and sulfa drugs. During the growth of bacteria, they generate acid, thus, altering the agar pH. The existence of antibiotic traces detected merely by comparing colors. This kit requires only 100 µL of the milk sample and only 3 hours incubation. Thus, Delvotest® SP NT presented as an appropriate, speedy, simple to use, and low-cost alternative test for the detection of numerous antibiotics in milk products. It is also suitable for the simultaneous analysis of a large number of specimens applying a short and simple process. The previous literature reported several analytical procedures describing screening of antibiotic remains in milk [14,15]. These practices
utilized microbial assays and instrumental analysis. Even though analytical methods as HPLC induce precise data of the concentration of residual antibiotics, it needs costly apparatus and skilled investigators. Because of the speed and easiness of microbiological assays, they are applicable for the prescreening of probable antibiotics. Nevertheless, objectionable false negative or false-positive findings might arise if using microbiological testing.

The probable unfavorable impacts on human health produced by antimicrobial residues determined by computing the risk estimation. Normally, estimation of chemical risk involves four distinct steps; hazard recognition, hazard description/dose-response estimation, exposure evaluation, and risk depiction. Estimation of chemical risk has two extensively utilized concepts, which are; Hazard quotient (HQ) and risk quotient (RQ). The hazard quotient is utilized for the estimation of health risk whilst the risk quotient is employed in the assessment of environmental risk. This methodology is greatly favored for maintaining food safety to guarantee public health [15].

The current study aimed to perform prescreening of raw milk specimens to detect antibiotic residues by microbiological inhibitor test kits followed by confirmatory quantitative analysis by an HPLC/MS-MS technique. Furthermore, antibiotic residues in milk were correlated with risk estimation for human health. In this study, the novelty is the application of a microbiological method followed by confirmation chromatography for screening antibiotic residues in milk samples in the Arab region, including Egypt and Saudi Arabia for the first time, and the assessment of health risk associated with positive results.
2. Materials and methods

2.1. Milk samples

Two hundred bovine milk samples were gathered from different locations from Saudi Arabia and Egypt after approval from Institutional Review Board, Princess Nourah bint Abdulrahman University (IRB Log Number: 21-0296) as shown in (Table 1). Samples collected from Saudi Arabia were 100 samples from various markets in Riyadh. One hundred milk samples from Egypt were collected from five different Governorates (Cairo, Giza, Dakahlia, Gharbia, Sharkia, Kafr El Sheikh, and Qalyoubia). All milk samples from Saudi Arabia were pasteurized milk of different brands, while Egyptian milk samples were both raw and pasteurized milk of different brands. All samples were kept at 4°C and analyzed by microbiological test.

Table 1. Collection sampling plan for local raw and pasteurized milk products

| Sample type           | Country   | Sampling region                  | No. of samples |
|-----------------------|-----------|----------------------------------|----------------|
| Local raw milk        | Egypt     | Cairo Governorate                | 10             |
|                       |           | Giza Governorate                 | 10             |
|                       |           | Dakahlia Governorate             | 10             |
|                       |           | Gharbia Governorate              | 10             |
|                       |           | Sharkia Governorate              | 10             |
|                       |           | Kafr El-Sheikh Governorate       | 10             |
|                       |           | Qalyoubia Governorate            | 10             |
| Local pasteurized milk| Egypt     | Juhayna products                 | 5              |
|                       |           | Lamar Egypt products             | 5              |
|                       |           | Dina Farms products              | 5              |
|                       |           | Almarai products                 | 5              |
|                       |           | Beyti products                   | 5              |
|                       |           | Lactel products                  | 5              |
| Local pasteurized milk| Saudi Arabia | Almarai products  |                |
|                       |           | Full cream milk                  | 5              |
|                       |           | Low-fat milk                     | 5              |
|                       |           | Full cream laban                 | 5              |
|                       |           | Low-fat laban                    | 5              |
Ayran laban
Alsafi products
Full cream milk 5
Low-fat milk 5
Skimmed milk 5
Full cream laban 5
Low-fat laban 5
Nadec products
Full cream milk 5
Low-fat milk 5
Full cream laban 5
Low-fat laban 5
Saudia products
Full cream milk 5
Low-fat milk 5
Activia products
Full cream milk 5
Low-fat milk 5
Full cream laban 5
Low-fat laban 5

| Total Samples | 200 |

2.2. Delvotest SP-NT (Microbial inhibitor test)

Delvotest SP-NT is a non-specific microbiological test, performed to identify the existence of antibiotic residues in dairy milk. The principle of the test is agar diffusion, in which the agar test tubes contain a fixed standard count of Bacillus stearothermophilus spores, nutrient agar, and bromocresol purple as a pH indicator. Delvotest SP NT purchased from DSM Food Specialties located in Spain [16]. The sample was added directly to the agar surface (ampoules), then incubated at 64 °C for 3 h. After incubation, color is changed from purple to yellow due to a change in pH resulting from microbial metabolism. In the case of fermented milk, heating first for 10 min at 80 °C to remove natural inhibitors lysozyme and lactoferrin [17]. Test and data interpretation
carried out based on the manufacturer’s instructions. Visual interpretation of the test results evaluated as ‘negative’ (yellow) and ‘positive’ (blue or purple).

2.3. Chemicals

The antibiotic analytical standards; tetracycline HCl, oxytetracycline HCl, chlortetracycline HCl, doxycycline hyclate, nalidixic acid, norfloxacin, ofloxacin, enrofloxacin, ciprofloxacin, lomefloxacin HCl, erythromycin A, oleandomycin triacetate, Tylosin tartrate, tilimicosin, Josamycin, spiramycin, roxithromycin, lincomycin HCl, clindamycin, trimethoprim, sulfadiazine, sulfadoxine, sulfamethoxazole, sulfathiazole, sulfachlorpyridazine, sulfanilamide, and sulfamethoxazole, were obtained from Sigma–Aldrich (Seelze, Germany). The Arab Company for Gelatin and Pharmaceutical Products (Alexandria, Egypt) provided ampicillin and amoxicillin raw materials. Analytical grade Formic acid, disodium hydrogen phosphate dihydrate, citric acid monohydrate, and trichloroacetic acid were of analytical grade and were purchased from Merck (Darmstadt, Germany). Phosphoric acid was from Riedel-deHaën (Seelze, Germany). Preparation of the McIlvaine buffer was by adding 0.1 M citric acid hydrate to 0.2 M disodium hydrogen phosphate (60:40, v/v). The washing solution was prepared by blending water and methanol (95:5, v/v). Twenty percent trichloroacetic acid solution was made to precipitate protein. Regenerated cellulose membrane filters and syringe filters (Minisart RC25) with pore size 0.45 μm were from Sartorius-Stedim (Goettingen, Germany). The solid-phase extraction columns Chromabond ABC18 (C18) sorbent were purchased from Macherey-Nagel, Düren, Germany.
2.4. Equipment

Confirmatory chromatographic analysis was executed on an Agilent Technologies HPLC system 1260 (Agilent Technologies, USA). Detection using mass spectrometry was undertaken employing a triple quadrupole API 4500 (ABSciex, Canada), that operated in the positive electrospray ionization under selected reaction monitoring mode. The mass spectrometer settings used were as follows: dwell-time = 20 ms; resolution Q1 and Q3 = unit; nebulizer gas = 12 psi; curtain gas = 12 psi; collision gas = 8 psi; ion spray voltage = 5500 V; temperature = 400 °C.

Controlling the hardware and the data procurement and treatment were accomplished utilizing Analyst 1.6.3 Software (ABSciex, Canada). A vortex shaker from Heidolph (Schwabach, Germany), a TDL-60B Centrifuge (Anke, Taiwan), and BHA-180 T Sonicator (Abbotta Corporation, USA) were employed for the sample preparation and the extraction procedure.

2.5. Chromatographic Conditions

All conditions used were according to the reference method. LC analyses were executed using a Nucleodur MN-C18 column (150 mm × 4.6 mm i.d., 5 μm particle size), Macherey-Nagel, Düren, Germany. The mobile phase used was a gradient of parts A (water containing 0.2% formic acid) and B (acetonitrile containing 0.2% formic acid) at an oven temperature of 30°C with a flow rate of 0.3 mL/min. The gradient started with 90% of eluent A for 1 min, then reduced to 40% through 11 min. This composition was kept steady for 3 min, then raised to 90% of eluent A within 1 min.
2.6. Stock Standard Solutions

Preparation of stock standard solutions of all analytical standards was performed by accurately weighing the materials that were dissolved in methanol. But, for solubilization of quinolones, it is necessary to add 2% of a 2 M ammonium hydroxide solution to methanol solution. All stock solutions of concentration 1 mg/mL were stored in the refrigerator. Working standard mixed solutions of each group of antibiotics were prepared in a concentration of 20-fold MRL for tetracyclines, macrolides, lincosamides, and quinolones and 10-fold MRL for sulfonamides through dilution with the mobile phase (water and acetonitrile 90:10, v/v, with 0.2% formic acid). If no MRL existed, the concentration of the analyte in the mixture was 0.2 μg/mL.

2.7. Preparation of Samples

Preparation of milk samples was performed adopting a reference method [18]. 5 mL of each of suspected milk samples were weighed in a centrifuge tube. The samples were mixed with 100 μL of trichloroacetic acid solution 20% (w/v) and the mixtures were vortexed. 10 mL of McIlvaine buffer of pH 4.0 were added and the mixtures were vortexed for 1 min and then, subjected to centrifugation at 4000 rpm for 15 min. The supernatant was removed and filtered. The filtrate was then exposed to a solid-phase extraction procedure. First, conditioning of the SPE cartridges was made using 6 mL of methanol and 6 mL of water. The filtrate was then moved to the cartridge. The washing step was done with 6 mL of 5% methanol in water (v/v), the cartridges were then dried for 10 min. Then, elution of the analytes was done using 6 mL methanol, followed by vaporization to dryness in nitrogen. The residue was redissolved in 1 mL of the mobile phase (water and acetonitrile 90:10, v/v, with 0.2% formic acid) and analyzed.
2.8. Calculation of Hazard Quotient and Risk Estimation

The model of Hazard Quotient was utilized to estimate the risk of ingesting residues with milk. Hazard quotient is defined as the ratio of the prospective exposure to a material and the concentration where no harmful impacts are anticipated.

\[
\text{Hazard Quotient} = \frac{\text{Estimated Daily Intake (EDI)}}{\text{Acceptable Daily Intake (ADI)}}
\]

The estimated daily intake (EDI) was computed using the subsequent equation presented by Juan et al. [19].

\[
\text{EDI} = \frac{\text{Concentration of Residue in } \mu g \times \text{Daily Intake of milk in Kg/person}}{\text{Adult Body Weight (Kg)}}
\]

The mean level of residual antibiotics in raw milk was calculated. Then, the mean concentration and normal daily milk consumption based on 60 kg body weight were utilized for calculations. According to the data supplied by the Food and Agriculture Organization of the United Nations, The per capita availability of milk in Egypt was 96.98 ml/day [20].

Acceptable Daily Intake (ADI) is an approximated quantity of residue permitted to be consumed daily throughout a life expectancy with no noticeable health risk stated based on body weight. ADI of oxytetracycline is 0.03 mg/kg bw/day [21].

If the hazard quotient is lower than or equal to one, this implies insignificant hazard whilst if the value is greater than one, it indicated the possibility of harm [22].
3. Results

3.1. Delvotest SP-NT (Microbial inhibitor test)

In this study, the detection of antibiotic residues in milk samples was performed using a microbial inhibitor test and liquid chromatography-mass spectrometry. Results of Delvotest SP-NT revealed that 40 out of 200 tested samples showed no color change or partial color change, suggesting a positive result constituting 20% of the total samples as shown in (Fig 1). After that, all of the 40 positive samples were tested using HPLC-MS/MS method as a confirmatory test.

Fig.1. Results of Microbial inhibitor test (Delvotest SP-NT)

3.2. HPLC-MS/MS

Results of HPLC-MS/MS revealed that only 4 out of 40 suspected samples analyzed were found truly positive (10%) containing oxytetracycline residues. We could not find any of the other tested antibiotics from the rest of the suspected positive samples via HPLC-MS/MS analysis which were consequently, considered false positive. These positive results were detected in milk samples obtained from the farms in Egypt. The four positive samples contained 120, 132, 141, and 150 μg/Kg, thus, exceeding the MRL which is 100 μg/Kg [20]. It could be concluded that milk obtained from treated cows contained a residual antibiotic. The Chromatogram obtained because of the analysis is presented in (Fig 2).

Fig.2. Typical chromatogram of milk sample positive for oxytetracycline.
3.3. Hazard Quotient and Risk Estimation

The estimation of health risk for confirmed positive milk samples from dairy farms was conducted to verify the threats to consumers resulting from the intake of milk containing antibiotic residues as they were exceeding the MRL. The mean oxytetracycline concentrations in the tested milk samples were 120, 132, 141, and 150 μg/kg. HQ for detected oxytetracycline remains in milk samples from dairy farms was computed to estimate any health threats to consumers (Table 2). As the HQ for the detected residues of oxytetracycline in milk samples from farms was less than one, so it may be presumed that there were insignificant adverse impacts on the consumer health accompanying the intake of the investigated samples.

| Mean Concentration (μg/Kg) | Age Group | Body Weight* (Kg) | EDI   | ADI (3) | Hazard Quotient |
|-----------------------------|-----------|-------------------|-------|---------|-----------------|
| 120                         | Adults    | 60                | 0.194 | 30      | 0.0065          |
| 120                         | Children  | 10                | 1.164 | 30      | 0.0388          |
| 132                         | Adults    | 60                | 0.213 | 30      | 0.0071          |
| 132                         | Children  | 10                | 1.280 | 30      | 0.0427          |
| 141                         | Adults    | 60                | 0.228 | 30      | 0.0076          |
| 141                         | Children  | 10                | 1.368 | 30      | 0.0456          |
| 150                         | Adults    | 60                | 0.242 | 30      | 0.0081          |
| 150                         | Children  | 10                | 1.455 | 30      | 0.0485          |

Abbreviations: ADI, acceptable daily intake; EDI, estimated daily intake.
**4. Discussion**

Examining milk samples for detection of antibiotic residues in levels more than was set by community legislation needs low-cost testing methods. Due to their effectiveness, microbial inhibition methods were largely used instead of physical-chemical methods. Those methods have many advantages as they could be easily operated with no need for special training, simple equipment, and could detect a broad variety of antibiotic traces within a single test [23]. The most commonly used tests are microbiological tests using *Bacillus stearothermophilus* spores, Delvotest SP, Copan Test, Charm Farm-960 Test, and others [16]. In the current study, the test that is used for the detection of the antibiotic in the examined milk samples collected from both KSA and Egypt is Delvotest SP-NT. Non-specificity is the main limitation of these microbial assays [17]. Results of Delvotest SP-NT were assessed visually as purple and yellow colors, which are easy to visualize. However, the problem was for reading the samples containing intermediate concentrations of antibiotics that render the visual reading of the reaction more difficult [24,25]. In those samples, the agar medium appeared as a mix of purple in a yellow background indicating a doubtful positive result. Moreover, different milk types and different antibiotic mechanisms of action make it more challenging to assess due to their different colors [25]. Therefore, microbiological testing is less appropriate for conclusive analyses leading to debatable results (false positives). Another illustration of false-positive Delvotest results may occur due to incomplete milking and natural inhibitors in samples from freshly cows [26]. These results should therefore be confirmed by more specific and sensitive techniques, such as HPLC-MS/MS, which was used in this study to confirm the Delvotest results. In this study, the results of Delvotest SP-NT revealed that the positive samples were those collected from treated farm cows. HPLC-MS/MS
also confirmed that samples obtained from farms were positive for antibiotic residues. This may be due to collection of milk just after antibiotic administration, drug misuse, or bad hygiene [27-30]. HPLC-MS/MS revealed only four positive samples containing oxytetracycline residues as was reported in previous articles [31]. The low number of milk samples containing antibiotic residues may be due to the use of growth promoters by farmers rather than the use of antibiotics. The results of this study coincide with studies in Algeria that were done to detect antibiotics in milk. Hakem et al [32], detected no antibiotic residues in milk samples obtained from two Dairies Mitidja’s Farms using Delvotest SP NT. In another study, about 10% of bulk tank milk samples and 20% of untreated bovine milk were reported positive [33]. These results were higher than those observed in Algiers (9.87 %) provided by Ben-Mahdi & Ouslimani [34]. On the other hand, other studies ran against ours including, Zinedine et al.[27] in Morocco, Tarzaali et al. [35] in Mitidja, Aggad et al. [36] in the west of Algeria and Titouche et al. [37] in Tizi-Ouzu. Those detected a higher percentage of antibiotic residues in milk (57, 89, 29, and 46 %, respectively). The HPLC-MS results we obtained were close to those reported by Martins et al. [38] in Brazil, who found 1.76 % of antibiotic residues in milk samples. Other studies produced a positive ratio of more than 15 % as reported by Li et al. [39] in China and García et al. [40] in Spain reporting 28 %.

To determine the threat posed to consumers by milk containing antibiotic residues at levels exceeding the MRL, health risk estimations were conducted for confirmed positive milk samples collected from dairy farms. The mean oxytetracycline concentrations in the tested milk samples were 120, 132, 141, and 150 μg/kg. HQ for detected oxytetracycline remains in milk samples from dairy farms was computed to estimate any health threats to consumers (Table 2). HQ is defined as the ratio of the possible exposure to a material and the concentration where no harmful impacts
are noted. If HQ is less than or equal to one, the risk to human health will be insignificant, while if HQ is exceeding one, the consumer is subjected to the risk. As the HQ for the detected residues of oxytetracycline in milk samples from farms was less than one, so it may be presumed that there were insignificant adverse impacts on the consumer health accompanying the intake of the investigated samples. Comparable results were stated by Moudgil et al. [41], who assessed the dietary exposure to residual antibiotics detected in raw and commercial milk samples in Punjab, India. The study stated no toxicological threat to consumers accompanying the intake of the examined milk samples concerning the antibiotics under study. Similar conclusions were also described by Rahman et al. [22], where the estimated dietary exposure to residual antibiotics through milk in Bangladesh was lower than the toxicological standard value.

5. Conclusion

One of the most significant concerns affecting public health is antibiotic residues found in milk. According to this study, Egyptian and Saudi Arabian cow's milk contained low levels of antibiotics. Among 40 suspicious samples examined, only four (10%) tested positive for oxytetracycline residues. From the remaining suspected positive samples, none of the other examined antibiotics were detected by HPLC-MS/MS, and so this was deemed a false positive. These positive results were detected in samples obtained from the farms in Egypt. The four positive samples contained 120, 132, 141, and 150 μg/Kg, thus, exceeding the MRL which is 100 μg/Kg. Hereby, the milk collected from treated cows contained antibiotic residues. The occurrence of antibiotic residues in milk indicates the importance of further control of milk, which is tested using microbiological Delvotest SP-NT and confirmed with HPLC-MS/MS. We could
conclude that HPLC-MS/MS could be considered a reliable analytical method for determining whether milk contains multiple antibiotics.

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**Conflict of interest**

The authors did not declare any conflicts of interest.

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Supporting information

S1 Fig. Results of Microbial inhibitor test (Delvotest SP-NT)

S2 Fig. Typical chromatogram of milk sample positive for oxytetracycline.
S1 Table. Collection sampling plan for local raw and pasteurized milk products

S2 Table. Estimation of Human Health Risk based on hazard quotient for oxytetracycline residues through milk consumption in adults and children from dairy farms
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Dear editor:

I have incorporated all of your suggestions into my revision. They were very helpful. Thank you.

- Proofreading and copy-editing was done.
- The novelty of this work was added.
- Information about LC-MS/MS setting was also added.