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Comparative Survey of Sulfonamides in Chicken Liver in Tunisia and sample freezing effect on Charm II

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Abstract

The aim of this work was to investigate the occurrence of sulfonamides in chicken liver in Tunisia. A total of seventy-three chicken liver samples were collected and analyzed using the Charm II Rapid Test Kit in January 2020. The percentage of positive samples in chicken liver derived from farms, traditional, and industrial slaughterhouses was determined and reached respectively 60%, 90% and 28%. (Detected sulfonamides) levels where all above below the maximum residue levels. The maximum residue levels (MRLs) of 100 ppb is set by the Codex Alimentarius Commission (CAC). Moreover, the effect of freezing time on the results of the Charm II radio receptor assay technique of negative samples of chicken liver was studied. This work demonstrates the vital need for a more regulated supervision of the use of sulfonamides, particularly in farms and traditional slaughterhouses, in Tunisia. In addition, the results of the kit Charm II were shown to be affected by the storage period of samples at 4°C.

1. Introduction

Sulfonamides, also called Sulfa drugs, are antimicrobial agents most commonly used in veterinary practice to treat diseases to control and prevent infection and to promote growth and production efficiency [1-3]. Chemically, sulfonamides are organic sulfur compounds that contain SO_2NH_2 radical (Figure 1).

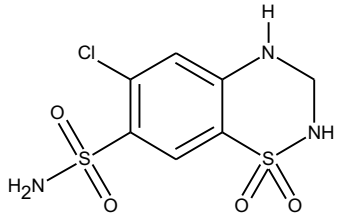

Figure 1. Chemical structures of sulfonamides

These veterinary medicines are soluble in polar solvents such as ethanol, acetone, acetonitrile and chloroform but insoluble in nonpolar solvents [4]. The four common sulfonamides are sulfadiazine, sulfamethazine, sulfamethoxzole and sulfaquinoxaline [5-6]. The extensive use of sulfonamides as a

result of their low cost caused the emergence of many sulfonamides resistant strains of bacteria [7-8]. Mainly, three genes (*sul*1, *sul*2 and *sul*3), encoding dihydropteroate synthetase (DHPS) with a low affinity for sulfonamides, mediate the resistance to these "High priority" veterinary antibiotics [9-10].
Screening methods like the Charm II radio receptor assay technique [11-14] have had an increasing success thanks to their ability identifying suspected samples at a considerably reduced costs and times. The Charm II test is based on the irreversible binding reaction between the functional groups of antibacterial and receptor sites on or within the cells of the added microorganisms. The test employs ^{14}C or ^{3}H radiolabelled antibacterial (tracer reagent) to compete for the binding sites. This competition for the receptor sites prevents the radiolabelled antibacterial from binding. Thus, the more radiolabelled compound binds, the less analyte (drug concentration) is in the sample. A scintillation counter measures the amount of tracer on the binding agent and compares with a control point [15].
The safety of sulfonamides to consumers has been questioned because of their toxicity [16]. The European Union (EU) (1990) [17] has set maximum residue limits (MRLs) for different food contaminants in a certain number of raw foods. Food products containing concentrations of antibiotics exceeding the established MRL are inappropriate for human consumption (2003) [18].
The (EU) Commission has established (MRL) of 100 $mg.kg^{-1}$ for the sum of all sulfonamides in target tissues (fat, kidney, liver and muscle) and milk from all food-producing species (European Commission, 2010) [19].
The purpose of this work was dedicated to determine sulfonamide residues in chicken liver by the screening method Charm II test and study the effect of freezing time on chicken liver negative samples.

2.Materials and Methods

The Charm II radio receptor assay was purchased from (Charm Sciences Inc. 659 Andover, Lawrence, MA USA) [20]. This kit contained MSU Multi-Antimicrobial Concentrate Standard, an MSU Extraction Buffer, a Tissue Performance Negative Concentrate, a M2 Buffer, tablet reagents and a scintillation fluid. Prepared stock solutions were stored in glass at 4°C.

2.1. Sampling and sample treatment

Seventy-three of chicken liver and muscle tissues were purchased from many traditional, industrial slaughterhouses and farms in the Bizerte region in Tunisia for testing sulfa drugs. Frozen chicken muscle and liver samples were allowed to thaw at room temperature (25°C) before processing. Forty ml of MSU extraction buffer, provided in the test kit, was added to 10 g of liver in a 50 ml centrifuge tube. The mixture was poured into a food processor and homogenized for 30–60 sec. The homogenate was poured back into the 50 ml centrifuge tube and incubated at 80°C for 45 min. The tube containing the incubated homogenate was placed in an ice water bath for 10 min then centrifuged at 1750 x g (33x100 rpm on Hettich Rotofix 32 centrifuge) for 10 min. The resulting supernatant was decanted into a clean 50 ml centrifuge tube and used for testing and the tissue pellets were discarded. Briefly, for the detection of any sulfonamide drug, two reagents are used: a [3H] labeled sulfamethazine, and a binding reagent (specific receptors in a microbial cell). Antibiotics in a sample compete with the tracer for receptor sites on the binding agents. When the binding reagent is
added to a sample with sulfonamides, the contaminating sulfonamide binds to the receptors attached to a cell. This prevents the [3H] sulfamethazine from binding to these sites. The detection reaction is stopped with centrifugation step, where unbound tracer is separated from bound tracer–binder complex, and analyzed in a scintillation counter for 1 minute to give a resulting count. The amount of [3H] sulfamethazine in the sample is measured using a scintillation counter. The pellet (containing tracer-

binder complex) collected after centrifugation was analyzed in the counter for one (1) minute to determine the count. High count results measured as counts per minute (cpm) reflect low antibiotic levels and the samples were considered negative for antimicrobial agents and low counts were considered positive for antimicrobial agents.

The data is simplified to a present/ absent result using a control point. The control point is the cutoff number between a negative and a positive result. This point is established by averaging the counts per minute for six negative controls and subtracting 20% (N=6)−20%=829. The more [3H] sulfamethazine bound, the less sulfonamide drug there is in the sample. The lower the result number (cpm), the higher the amount of contamination is in the sample.

The supernatant, obtained after the liver processing was diluted in a ratio of 1:4 with a negative control. The pH was then observed using a pH strip and adjusted to pH 7.5 using the M2 buffer provided within the kit. The same procedure used for liver samples was also applied to process muscle tissues; however, the supernatant was not diluted. The final extract was tested for sulfonamides using the Charm II protocol provided by the manufacturer. All antimicrobial agents were detected qualitatively. Samples with counts per minute (cpm) less than or equal to the control point were considered "suspect". Suspect samples were retested with the negative control and the positive control as prescribed by the manufacturer. If the retested sample counts were still less than or equal to the control point and the control test results were in the expected range, the sample was considered as positive.

3.Results and discussion

3.1 Sulfonamides detection using the kit Charm II

Thirty samples of chicken livers were collected from weekly souks and small markets in Bizerte at Tunisia for detection of residues of sulfonamides. The monitoring of these veterinary antibiotics was done using the Charm II antibiotic test system. The Charm II kit is a highly reproducible method for the screening of antibiotic residues in chicken and other matrices [20,21]. Figure 2 represents the experimentally determined detection limits of sulfonamides in chicken by means of the Charm II kit are 100 μg.kg^{-1} (CCA 2017, Charm Sciences) [20].

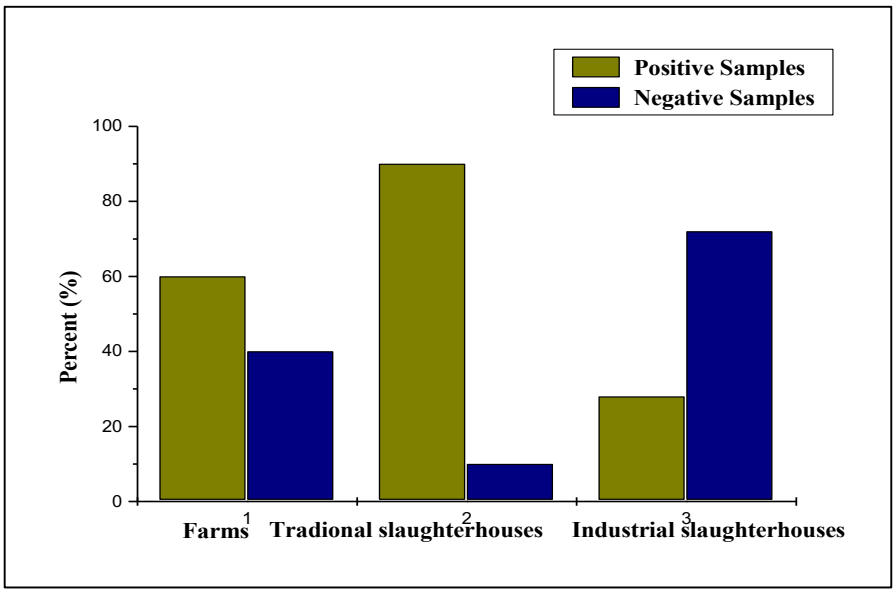

Figure 2. Comparison of sulfonamides antibiotic residues in chicken liver obtained from farms, traditional and Industrial slaughterhouses.

It can be seen in Figure 2 that the percentage of positive samples in chicken liver from farms, traditional, and industrial slaughterhouses was respectively 60%, 90% and 28%. Those results suggest that residues of sulfonamides in chicken liver are higher in samples from farms and traditional slaughterhouses.

In the same context, related to the presence of antibiotic residues in foodstuffs of animal origin, many research studies were reported in literature. For example, Abiola et al. revealed a contamination rate of 54% on chicken livers in both Dakar and Senegal regions [22]. Moreover, In Saudi Arabia, Al-Ghamdi et al found 69.7% antibiotic residues in chicken flesh [23]. Also, In France, comparable studies were also carried out by Valdebouze et al who found a level of 10% of residues of antibiotics with antibacterial activity in the meat of chickens sold commercially [24]. The same observation indicated that the contamination rates of sulfonamides in chicken liver samples were higher than authorized limits as is the case in the USA, Italy, Malaysia and many other Asian countries [25-28].

This is a situation encountered throughout the world. In our study, it is legitimate to conclude that the withdrawal time of antibiotics before slaughter has been ignored. Consequently, proper management strategies for controlling the usage of antibiotics, monitoring their withdrawal time and screening their residues represent urgency. Within these results, presently, no information is available on the number of false negatives and false positives because no other screening tests were run and samples were not analyzed by quantitative chemical methods. For instance, confirmatory analytical techniques as HPLC can be done in order to verify the results of screening tests. The MRL of 100 ppb as defined by the Codex Alimentarius [29,30] constitutes the sum of the concentrations of all the sulfonamide molecules present in liver tissue. As a result, we analyzed a negative sample doped with 20 ppb, 30 ppb, 50 ppb, 70 ppb and 100 ppb of Sulfamethazine. Obtained results were illustrated in Figure 3.

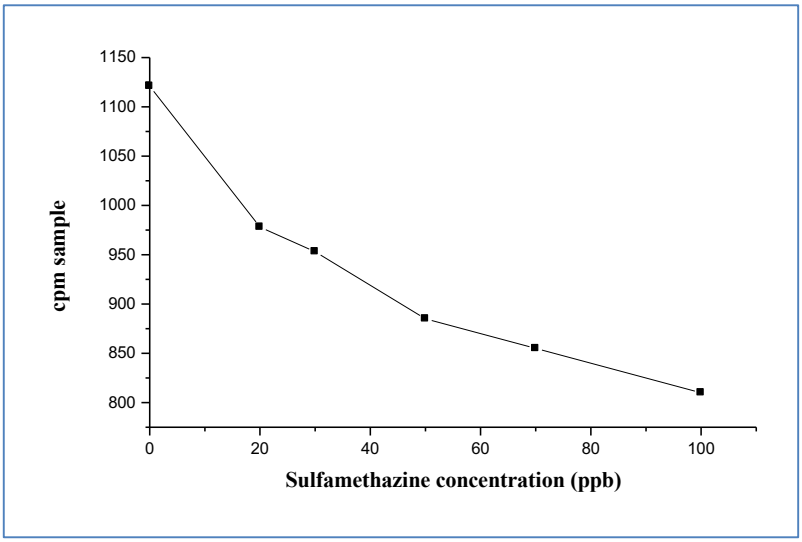

Figure 3. Correlation between the Sulfamethazine concentration and the counts per minute (cpm) of an analyzed chicken liver sample using the Charm II kit.

The examination of Figure 3 illustrating the cpm values as a function of as doped sulfamethazine concentration, shows that after doping with 20 ppb, 30 ppb, 50 ppb and 70 ppb of sulfamethazine, the results appear negative and (as long as their cpm values remain above the control point equal to 829). However, when the samples where doped with 100 ppb sulfamethazine concentration, the cpm value was lower the control point, consequently the sample was declared positive. Following these results, it can be concluded that the limit of detection of sulfonamide residues in liver tissue using charm II, as screening method is equal to 100 ppb. Thus, charm II test is unable to detect the presence of sulfonamides in chicken liver at concentrations below 100 ppb.

3.2 Effect of freezing:

During this work, we studied the freezing parameter which is an important parameter to verify it influence on the response. In this context, we have putted six samples of chicken liver previously declared negative at a temperature of 4 °C for four months and we reproduce the same investigation. All results are listed in Table 1.

Table1: *Study of the effect of freezing time on negative samples.*

Samples	**1**	**2**	**3**	**4**	**5**	**6**
1st day	Negative	Negative	Negative	Negative	Negative	Negative
After 1 month	Negative	Negative	Positive	Negative	Positive	Positive
After 2 months	Positive	Negative	Positive	Negative	Positive	Positive
After 3 months	Positive	Negative	Positive	Negative	Positive	Positive
After 4 months	Positive	Positive	Positive	Positive	Positive	Positive

Results show that the detection rate of sulfonamides in chicken liver samples changed from 0 to 100%. Indeed, negative results start changing to positive ones after four weeks of freezing at 4°C. Also, it is important to notify that all negative samples were changed to positive after four months of freezing.

These results can be explained that during storage, microorganisms or enzymes can lead to the decomposition of proteins or other molecules chicken liver [31-33]. In addition, Autochthonous chicken microbiota [34-36] and external microbial contaminations following the slaughter may cause the appearance of new antimicrobial-like molecules and a decomposition of inhibitory molecules of the Charm II kit and consequently may explain the results of Table 1.

Conclusion

There is a potential sulfonamides risk in chicken liver products, particularly from farms and traditional slaughterhouses, in the Tunisian market. Moreover, whether the screening method Charm II is sufficiently specific evaluated through the proportion of healthy livers who were correctly screened as not containing sulfonamide residues (the proportion of negatives that were correctly detected) for accurate sulfonamides detection in liver of chicken remains questionable as the results of the kit were affected by the storage period of samples at 4°C.

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