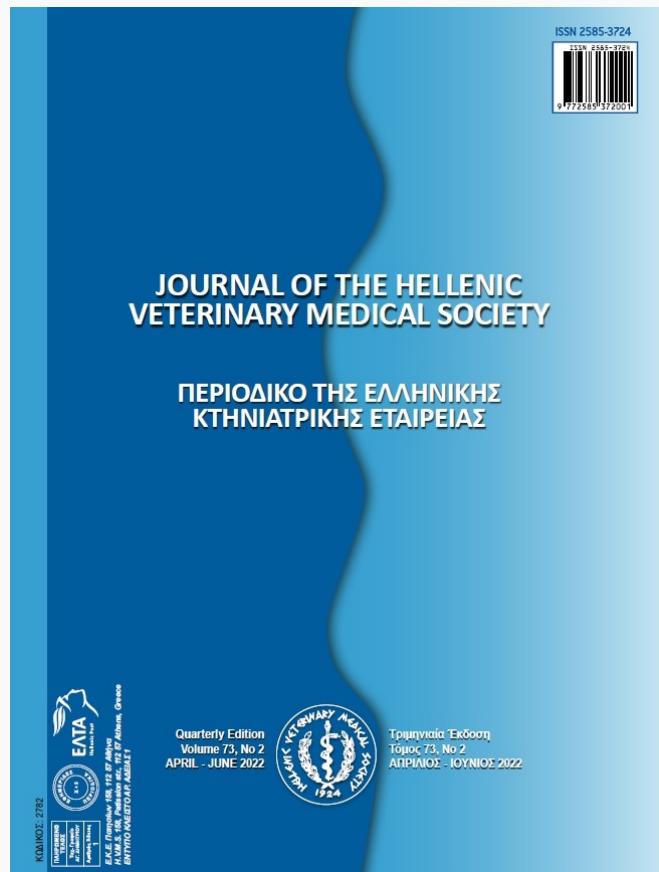


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Detection of antimicrobial residues in bovine meat marketed at Tizi-Ouzou area (Algeria), by a microbial screening assay

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ABSTRACT: The incidence of antimicrobial residues in bovine meat was determined using a microbiological method. Two hundred and nine samples of bovine meat (imported: 153; locally: 56) were collected through various retail outlets in Tizi-Ouzou area, Algeria. The collected samples were tested according to the European Union four-plates method (EU4pt), using *Bacillus subtilis* and *Micrococcus luteus* ATCC 9341. Only 60 (34/56 local meat and 26/153 imported meat) samples were free from antibiotics residues. Higher prevalence of residues was observed in imported bovine meat (83.01%) than local produced bovine meat (39.29%). High frequencies of contamination for all classes of antibiotics were observed in imported meat rather than local produced meat. From this latter, contamination with β -lactams and/or tetracyclines and sulfonamides was observed in 23.21% and 19.64% respectively. Sulfonamides were the most detected residues in the imported meat with 81.05%. 59.73% of samples were contaminated at least with two families of antibiotics residues, but in proportion that significantly ($p<0.05$) differ between imported (67.71%) and local produced meat (13.64%). Besides, one antibiotic residue family was detected in most (86.36%) of the contaminated bovine meat produced in Algeria. Our results confirm the non-respect of the withdrawal period between the treatment and slaughter of bovines. For this, further procedures for rational use of antibiotics in livestock and food quality control are required.

Keywords: antimicrobial residues; bovine meat; *Bacillus subtilis*; *Micrococcus luteus*; withdrawal periods.

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INTRODUCTION

In veterinary husbandry, antibiotics are used as therapeutic agents, for the treatment and control of many types of infections, or, in some countries, as growth promoters, to improve the efficiency of food utilization and weight gain (Ruiz & Alvarez-Ordóñez 2017). However, behavioral practices such as over use of antibiotics and lack of understanding about drug usage can lead to food contamination. The residues of veterinary drugs or its metabolites in meat and other foods of animal origin may cause adverse toxic effects on consumers' health, such as hypersensitivity, gastrointestinal disturbance and neurological disorder (Reig and Toldrà 2008; Chen et al. 2019). Another aspect of the problem is the fact that the unscrupulous use of antibiotics in animal production can increase and select multi-drug resistant bacteria, which can be transmitted to humans through the food consumption, environment or by direct contact through affected meat (Lee et al. 2000; Vishnuraj et al. 2016). To protect food safety and consumer health, Maximum Residue Limits (MRL) have been established by the European Union for pharmacologically active compounds (Arslan-Acaröz and Sözbilir 2020). In the same direction, the European Union (EU) has revised the technical criteria that must be applied in the screening and confirmatory analysis of these residues in foods of animal origin, also introducing the parameter called "minimum required performance limit (MRPL) (Moga et al. 2021).

There are two different methods for the determination of antibiotic residues: confirmatory and screening methods. The confirmatory methods included immunoassays, capillary electrophoresis, high-performance liquid chromatography, gas chromatography, and liquid chromatography-tandem mass spectrometry (LC-MS/MS) (Acaroz et al. 2020). All of these methods are expensive and time consuming and require personnel and adequate laboratory (Bacanlı and Başaran 2019). Screening methods are classified as microbiological assays and immunoassays. Microbiological assays are qualitative or semi-quantitative methods, based on a specific reaction between a susceptible organism (generally bacteria) and the antibiotic present in the sample. Some advantages of these assays are their reliability, cost effectiveness and simplicity (Chàfer-Pericàs et al. 2010). The most widely used method for the detection of antibiotic residues in animal originated foodstuffs is the microbial inhibition method. This method is not only cost effective but also able covers a large number of antibiotics in a

single test run (Vishnuraj et al. 2016). Most of the microbiological inhibition tests with agar diffusion are based on inhibition-diameter measurement. In these tests, samples were applied to plates of agar media inoculated with bacteria spores. Diffusion of an antibacterial substance was shown by the formation of inhibition zones (Chàfer-Pericàs et al. 2010). First of all, the European Union four-plate method (EU4pt) was assessed. This method comprises three plates of agar medium inoculated with *B. subtilis* BGA spores at pH 6, 7.2, and 8, and a *Kocuria rhizophila* (formerly known as *Micrococcus luteus*) ATCC 9341 plate at pH 8 (Pikkemaat 2009; Pikkemaat et al. 2011).

In Africa, livestock play an important role in socio-economic conditions, as many peoples depend on livestock for their income and livelihood. However, the use of antimicrobials in Africans countries remains unregulated, which may contribute to the contamination of foods of animal origin by antimicrobial residues (Van et al. 2020). In Algeria, very little is known about the usage of antibiotics in animal husbandry. Studies conducted by Ben Mahdi and Oussilmani (2009), Titouche et al (2013) and Layada et al (2016) indicated that antibiotic residues may be present at a high level into raw milk. However, few data were available concerning antibiotic residues in meat. Therefore, the main objective of the present survey is to seek for the eventual presence of antibiotic residues in meat marketed at Tizi-Ouzou area in order to assess the hazard health associated with the consumption of this animal food product.

MATERIALS AND METHODS

Collection of samples

The present study was carried out during 2018 and 2019 at the province of Tizi-Ouzou in Algeria. A province which is located on the central coast of Algeria (between 36°28' and 36°55' latitude north; 3°45' and 4°31' east). A random sampling methodology was followed for this work. A total of 209 samples of bovine meat were collected including local bovine meat (56) and imported bovine meat (153) from local markets and butchers. The imported frozen bovine meat comes from two countries, Brazil and Uruguay.

After sterilization of sampling equipment, 150 g of meat was taken in aseptic condition and put in a small bag. Each sample was packaged as a unit in each sterile plastic bag, carefully sealed and labelled. Each label includes code sample, origin, date and place of sampling. The samples were transported in the same

day to the laboratory under refrigeration. Using a scalpel, several slices of meat with 8 mm of diameter and 2mm of thick were taken and placed into Petri dishes and stored in frozen until they were ready to be analyzed.

Laboratory analysis

In this study, EU four-plates method (EU4pt) (AFS-SA, LMV/90/01) in which three plates of agar medium inoculated with *B. subtilis* BGA spores at pH 6, 7.2, and 8, and a *Micrococcus luteus* ATCC 9341 plate at pH 8 were used to screen the antibiotic residues in meat samples. The performance of this method is the ability to detect antibiotics levels above recommended maximum residues limits (MRLs) for various families of antibiotics including β -lactams, tetracyclines, chloramphenicol, macrolides, aminoglycosides, sulfonamides and quinolones. This method is applied to butcher animals and poultry meat.

Briefly, the test procedure was performed using *Bacillus subtilis* BGA spore and *Micrococcus luteus* ATCC 9341 strain as organisms test. A 0.5 Mc Farland's standard suspension of these organisms was prepared in 10 mL of broth brain heart infusion (Conda Pronadisa, Spain). Broth suspension of each of the test organism was adjusted with sterile physiological saline solution in order to obtain the turbidity equivalent of 3×10^8 cells/mL. This suspension was used to inoculate the surface of four test agar plates (Conda Pronadisa, Spain) prepared at different pH (*Bacillus subtilis* at pH 6; 7.2; 8 and *Micrococcus luteus* at pH 8). Two cylindrical pieces of meat were placed in diagonally opposite positions on each of the four seeded test plates. Trimethoprim (0.2 μ g/mL) was incorporat-

ed into medium at pH 7.2 in order to increase the sensitivity of the detection of sulfonamide residues. The *Bacillus subtilis* plates were incubated at 30°C for 18 - 24h, while the *Micrococcus luteus* plate was incubated at 37°C for 18 - 24h. After incubation, a positive test result was recorded when both meat discs on any plate gave a continuous annular zone of inhibition of not less 2mm across. In parallel, standard solutions of penicillin and erythromycin (Sigma Aldrich, Germany) were used as positive control. The different families of antibiotics detected by this method are listed in table 1

Statistical analysis

Chi-square tests, with Yates' continuity correction when needed and nonparametric tests allowing independence analysis between random variables were used. P <0.05 was considered statistically significant.

RESULTS

A total of 209 samples of bovine meat were collected for antibiotics residue analysis. Only 60 (34/56 local produced meat and 26/153 imported meat) samples were negative for antibiotics residues. Higher prevalence of residues was observed for the imported bovine meat (83.01%) than the local tested bovine meat samples (39.29%) (Table 2).

From the antibiotics family's standpoint, the residues of sulfonamides and β -lactams and/or tetracyclines were the most frequently diagnosed in the all-tested samples. High frequencies of contamination for all classes of antibiotics were detected from imported meat rather than local produced meat. From this latter, contamination with β -lactams and/or tet-

Table 1. Antibiotic target families depending on the microorganism test and pH of agar plate

Antibiotic target family	Microorganism test	Plate N°	pH of the medium	Incubation temperature
β -Lactams and/or tetracyclines	<i>Bacillus subtilis</i>	1	6.0	30°C
Sulfonamides	<i>Bacillus subtilis</i>	2	7.2	30°C
Aminoglycosides	<i>Bacillus subtilis</i>	3	8.0	30°C
Macrolides and/or β -Lactams	<i>Micrococcus luteus</i>	4	8.0	37°C

Table 2. Number of meat samples and prevalence of contamination by antibiotic residues

Analysis for antibiotic residues	Total No. (%)	Bovine meat origin	
		Locally produced meat No. (%)	Imported meat No. (%)
Number of tested samples	209	56	153
Free from antibiotic residues	60 (28.70)	34 (60.71)	26 (16.99)
Contaminated with antibiotic residues	149 (71.29)	22 (39.29)	127 (83.01)

Table 3. Meat contamination with residues according to the families of tested antibiotics

Analysis for antibiotic residues	Total No. (%)	Bovine meat origin	
		Locally produced meat No. (%)	Imported meat No. (%)
Number of tested samples	209	56	153
β-lactams and/or tetracyclines	100 (47.84)	13 (23.21)	87 (56.86)
Sulfonamides	135 (64.59)	11 (19.64)	124 (81.05)
Aminoglycosides	64 (30.62)	2 (3.57)	62 (40.52)
Macrolides and/or β-lactams	73 (34.92)	3 (5.35)	70 (45.75)

Table 4. Frequency of contamination of meat by antibiotic residues

Analysis for antibiotic residues	Total No. (%)	Bovine meat origin	
		Locally produced meat No. (%)	Imported meat No. (%)
Contaminated samples	149	22	127
With one family of antibiotic	60 (40.26)	19 (86.36)	41 (32.28)
With two families of antibiotic	33 (22.14)	3 (13.64)	30 (23.62)
With plus of two families of antibiotic	56 (37.58)	0	56 (44.09)

racyclines and sulfonamides was observed in 23.21% and 19.64% of samples respectively. Sulfonamides were the most detected residues in the imported bovine meat with 81.05% of the tested samples (Table 3).

Upon the frequencies of antibiotics residues detection in the same samples, our analysis revealed that 89/149 of samples (59.73%) were positive for two or more families of antibiotics residues, but in proportion that significantly ($p<0.05$) differ between bovine imported meat (67.71%) and bovine meat produced locally (13.64%). Furthermore, only one antibiotic residue family was observed in most (86.36%) of the contaminated bovine meat produced in Algeria (Table 4).

DISCUSSION

The most widely used method for the detection of antibiotic residues in animal originated foodstuffs is the microbial inhibition method. This method is not only cost effective but also able to cover a large number of different antibiotics in a single test run (Pikkemaat 2009; Vishnuraj et al.2016). Two main test formats can be distinguished: the tube test and the multi-plates test. A plate test consists of a layer of inoculated nutrient agar, with samples applied on top of the layer, or in wells in the agar. Bacterial growth will turn the agar into an opaque layer, which yields a clear growth-inhibited area around the sample if it contains antimicrobial substances (Pikkemaat 2009).

The EU4pt was developed for detection of residues in meat. Unlike to others test, the four plates method is based in combination of pH conditions, which consequently, promote or inhibit the activity of antibiotics. The pH of medium affects the activity of certain antimicrobial substances. For examples, the activity of aminopenicillins and tetracyclines is increased in acidic pH, and the activity of macrolides, quinolones and aminoglycosides in alkaline pH (Yamada et al.1981; De Zutter et al.1985). The mechanisms of the effect of pH on antimicrobial activity are not completely understood and inconsistent from drug to drug. Moreover, it is based on the sensitivity or resistance of the organism test to a various antibiotic. For this, the combination of these factors makes possible a rough identification of antibiotics or antibiotics groups (Karraouan et al.2009).

In this study, a high prevalence rates of drug residues in imported and locally meat ranged from 39.29% to 83.01% were observed. However, the lowest level of contamination was reported in local meat than in imported meat. Our results corroborates with those of other authors who indicated the presence of antibiotic residues in chicken and beef meat (Donkor et al.2011; Ur-Rehman and Jabbar 2013; Hakem et al.2013; Ghasmi et al.2014; Ramatla et al.2017; Ezenduka2019; Manzoor et al.2019). The high level of contamination of our meat samples can probably be explained, by the overuse of antibiotics in animal production to treat and prevent against specific diseases, both in the therapeutic setting recommended by

the veterinarian but also in the self-medication. However, the non-observance of withdrawal requirements is probably the main factor of contamination of meat by antibiotics residues (Kabir et al.2004; Donkor et al.2011). As known, poor practices play a major role in contributing to antimicrobial residues in food of animal origin (Van et al.2020). Donkor *et al* (2011) reported several risk factors which contribute to the contamination of food of animal origin with drug residues, including lack of veterinary consultation by farmers in drug administration and the non-observance of withdrawal periods following drug administration. The study conducted by Njoga *et al* (2018) in Nigeria showed that farmers (53.3%) administered unprescribed antimicrobials and observance of withdrawal period was ignored in the majority (65%) of the farms. The widespread antibiotic use drives the emergence of antibiotic-resistant organisms (AROs) in food-producing animals, including organisms that can cause diseases in humans, such as enterococci, *Escherichia coli*, *Campylobacter* and *Salmonella* (Patel et al.2020).

In this study, high levels of contamination were accorded to β -lactams, tetracyclines and sulfonamides both for local and imported bovine meat. Our results are in agreement with those of other authors who indicated that β -lactams, tetracyclines and sulfonamides were present in high levels in milk and meat samples (Hakem et al.2013; Ghasemi et al.2014; Agmas and Adugna2018; Njoga et al.2018; Baazize-Ammi et al.2019). However, Jammoul and El Darra (2019) reported that ciprofloxacin (quinolones) represents the highest occurrence percentage (32.5%) in chicken meat samples in Lebanon. As reported by Ben *et al* (2019), sulfonamides and tetracyclines were present at higher concentrations and detection frequencies in meat products than quinolones, while aminoglycoside and β -lactams were also commonly detected in meat products. Many antibiotic classes used in humans (medically important) are currently utilized in the beef, dairy, pork, and poultry industries. Antibiotics commonly used for the treatment, prophylaxis and growth promotion of food animals include doxycycline, colistin sulfate, neomycin, tetracycline, enrofloxacin, ciprofloxacin and amikacin (Bungau et al.2021; Hassan et al.2021). In 2018, tetracyclines accounted for 66%, penicillins for 12%, macrolides for 8%, sulfonamides for 5%, aminoglycosides for 5%, lincosamides for 2%, cephalosporins for 1%, and fluoroquinolones for <1% of antibiotic sales for livestock (Patel et al.2020). Sulfonamides and β -lactams

were used to treat both protozoal and bacterial infections (Agmas and Adugna2018).

Results of our study showed that imported meat samples were more contaminated than local meat samples, which can be explained by the intensive use of antibiotics in these farm animals. The increase of antimicrobial consumption is due to the growing numbers of animals raised for food production coupled with increase in consumer demand for livestock products including meat and meat products (Van Boekel et al.2015). In 2010, China (23%), the United States (13%), Brazil (9%), Germany (3%), and India (3%) were the five countries having substantial shares of global antimicrobial consumption in food animal production. At the present time, China and Brazil are among the large scale consumers of antimicrobials (Ibrahim et al.2020).

In our study, it was not possible to quantify the antibiotic residues in meat samples. For this, it is difficult to measure the actual risk of antibiotic residues to consumers. As reported, the microbiological methods are basic screening methods for the detection of antimicrobial residues in foods. These methods are not only cost effective but able to cover a large number of different antibiotics in a single test run (Vishnuraj et al.2016). However, these methods have some disadvantages such as lack of specificity and the required long incubation time (Bacanli and Başaran 2019). For this, quantitative methods, such as HP-LC were required to quantify the drug residues in food samples.

CONCLUSION

This study exposed a potentially serious public health problem for consumers due to the presence of antimicrobial residues in meat analyzed samples, which denotes a higher intensity of antibiotic use and the non-respect of withdrawal periods by farmers. For this, good farm management practices, including routine vaccinations, good nutrition, good farm hygiene, and implementation of biosecurity practices are effective measures to reduce the use of antibiotics in livestock. Continuous surveillance for monitoring and control on the use of veterinary drugs are needed to ensure food safety.

CONFLICT OF INTEREST

The authors declare that they have no conflict of interest.

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