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Screening of antibiotics residues in chicken meat by four plates test in Eastern Morocco

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ABSTRACT

Antibiotics have been instrumental in the development of avian industry worldwide. They are used to protect the intensive farming against various diseases, maintain an animal welfare state and achieve significant weight gains. However, the massive and irrational use of antibiotics in animal production chains is incriminated in public health problems mainly the selection of multi-resistant bacterial strains and the presence of antibiotics residues in foodstuff of animal origin. The objective of this work was to evaluate the contamination of 204 samples of broiler's meat by antibiotics residues using the Four Plates Test. Samples were collected from different traditional slaughterhouses in the region of Eastern Morocco during12 months (from January to December 2014). The results show that 27.45% of the samples were positive to the Four Plates Test with 9.80% could contain β-lactams and/or tetracyclines residues, 9.80% could contain sulfonamides residues, 5.39% could contain aminoglycosides residues and 15.69% could contain macrolides and/or β-lactams residues. Positive samples recorded during the summer season (July, August and September) constitute 42.9% of total positive samples of the study year. These alarming results, call for more vigilance regarding antibiotics residues in foodstuffs of animal origin to protect public health.

KEY WORDS
Antibiotics; Antibiotics residues; Broiler; Four Plates Test; Eastern Morocco.

INTRODUCTION

In poultry farms, antibiotics (ABs) are used in therapeutic, prophylactic, metaphylactic and zootechnical purposes. They permit to control and prevent diseases, keep animal welfare and ensure economic benefits of these farms [24,25]. If therapeutic use is well accepted when we should treat sick animals, preventive and zootechnical use of ABs as growth promoters in healthy animals is strongly implicated in public health issues [3,4,25]. Indeed, the presence of ABs residues in foodstuffs of animal origin, resulting from non-respect of withdrawal period, can cause adverse allergic reactions in consumers and favour the selection of multi-resistant bacterial strains (MRBS) [2,9,12]. Thus, it is essential to better control therapeutic practices in intensive farming especially in poultry to ensure the consumer healthy meat without ABs residues [9].

Several technics were developed for screening ABs residues in different animal tissues and foodstuffs of animal origin. The Four Pates Test (FPT) is the official method for detecting ABs residues in waterfowl fatty livers, red meat and poultry meat in many countries [1]. It is based on the ability of certain residues belonging to five families of ABs to inhibit the growth of *Bacillus subtilis* and *Micrococcus luteus* under different culture conditions [6].

The present work aims to detect possible antibiotics residues into 204 samples of broiler meat collected from traditional slaughterhouses in Eastern Morocco during twelve months by the Four Plates Test. This is the

first academic study in Eastern Morocco which assesses contamination of broiler meat by antibiotics residues in different seasons of the year.

MATERIALS AND METHODS

The FPT was performed and validated by the French Agency of Food Safety (AFSSA, LMV/90/01. V7). Tryptone Soya Agar (TSA) and Nutrient Agar (NA) were procured at Biokar. Testagar pH6.0 (TA6), Testagar pH7.4 (TA7) and Testagar pH8.0 (TA8) were obtained from Merck, *Bacillus subtilis (BS)* and *Micrococcus luteus (ML)* from Microbiologics. Erythromycin (ERY), Penicillin G sodium (PG) Dihydrostreptomycin sesquisulfate (DHS), Trimethoprim (TMP), Sulfadimerazine (SLF), Sodium chloride (NaCl), D-(+)-Glucose, Manganese sulfate (MnSO4), Ethanol, Sodium hydroxide (NaOH) and Acetic acid were purchased from Sigma Aldrich. A punch of 8 mm diameter and sterile Whatman paper discs of 8 mm diameter were also used in this test. The sporulation medium (Findey and Field) was prepared using 15 grams of NA, 5 grams of D-(+)-Glucose and 0.03 grams of $MnSO_4$ in 1000 ml of distilled water. Lyophilized strains of *BS* and *ML* were rehydrated separately and cultured in TSA for 24 hours at 30°C (*BS*) and at 37°C (*ML*). Thus, *BS* was cultured on the sporulation medium Findey-Field at 30°C for 5 days. Spores were harvested in physiological saline (0.85% NaCl), counted and stored at 4°C after three successive washings (centrifugation at 4400 g and recovery of the pellet in saline). *ML* culture is dissolved in 0.85% NaCl before being counted and stored at 4°C. The enumeration is necessary to calculate the dilution that gives a final concentration of 5.10^4 CFU/ml for both test strains. ABs solutions were prepared with sterile distilled water at different concentrations: 5µg/ml (TMP), 0.2 IU/ml (PG), 5µg/ml (DHS), 20µg/ml (SLF) and 0,25µg/ml (ERY). To increase their solubility, ERY is previously dissolved in Ethanol, SLF in NaOH 0.1N and DHS in 5% Acetic acid. Once the culture media (TA6, TA7 and TA8) were prepared and sterilized, they were kept in their liquid state in a Bain-marie at 44°C before adding spores, bacteria and TMP according to the model presented in Table 1. These culture media containing sensitive germs were cast in Petri dishes 90mm previously annotated at 5 ml medium per dish.

Samples were taken from 204 traditional slaughterhouses in the region of Eastern Morocco (120 from Oujda, 12 from Nador, 12 from Berkane, 12 from Driouech, 12 from Taourirt, 12 from Jerada, 12 from Figuig and 12 from Guercif) over a period of 12 months from January to December 2014.The sample was stratified random at 4 samples per week and one sample per slaughterhouse. Each sample corresponds to a piece of 100 grams of broiler breast taken just after slaughter and stored at -18 ° C.

The test was made on batches of 9 samples. Before starting the test, samples were placed at room temperature for 3 minutes. Using a punch, a core of 8 mm diameter was taken from each sample then cut into discs of 2 mm. Two disks of the same sample were placed diametrically opposite in each of the four plates. Finally, sterile Whatman paper discs of 8 mm diameter were deposited in the center of each plate and soaked with 10µl of positive control antibiotic as indicated in Table 1. Results were read after 24 hours of incubation at 30°C for dishes containing *BS* and 37°C for those containing *ML*. The positive control exhibited an annular inhibition zone greater than 4 mm diameter. The samples are considered as positive if we observe an inhibition zone greater than 2 mm surrounded them.

RESULTS

The obtained results in Table 2 show that 27.45% of analysed samples were positive to the FPT. Globally, 9.80% of these samples containβ-lactams and/or tetracyclines residues, 9.80% contain sulfonamides residues, 5.39% contain aminoglycosides residues and 15.69% contain β-lactams and/or macrolides residues.

The results by quarter show that 27.45% of collected samples in winter (1^{st} quarter) were positive of which 9.80% containing β-lactams and/or tetracyclines, 7.84% containing sulfonamides, 5.88% containing aminoglycosides and 15.69% containing β-lactams and/or macrolides. In spring (2^{nd}quarter), 15.69% of samples were positive of which 3.92% containing β-lactams and/or tetracyclines, 3.92% containing sulfonamides, 7.84% containing β-lactams and/or macrolides and no sample contained aminoglycosides residues. Autumn samples (3^{rd} quarter) were the most contaminated seeing that 47.06% of them were positive of which 19.61% containing β-lactams and/or tetracyclines, 21.57% containing sulfonamides, 11.76% containing aminoglycosides and 27.45% containing β-lactams and/or macrolides. Finally, 19.61% of winter samples (4^{th}quarter) were positive of which 5.88% containing β-lactams and/or tetracyclines, 5.88% containing sulfonamides, 3.92% containing aminoglycosides and 11.76% containing β-lactams and/or macrolides. However, of the 204 tested samples, 5 samples were positive to 4 tests simultaneously, 2 samples were positive to three tests at the same time and 8 samples were positive to two tests at once.

Table 1: Sturt-up conditions of the Four Plates Test

	Plate 1	Plate 2	Plate 3	Plate 4
Culture medium (pH)	Testagar (6)	Testagar (7,4)	Testagar (8)	Testagar (8)
Sensitive germ	*Bacillus Subtilis*	*Bacillus Subtilis*	*Bacillus Subtilis*	*MicrococcusLuteus*
Temperature of culture	30°C	30°C	30°C	37°C
Added antibiotic	-	Trimethoprim	-	-
Positive control	Penicillin G	Sulfamerazine	Dihydrostreptomycin	Erythromycin
Researchedresidues	β-lactams and/or Tetracyclines	Sulfonamides	Aminosides	β-lactams and/or Macrolides

Table 2 : Percentages quarterly distribution of positive broiler meat samples to the Four Plates Test in Eastern Morocco between January and December 2014 (n = 204).

	Bacillus Subtilis pH 6	*Bacillus Subtilis* pH 7,4	*Bacillus Subtilis* pH 8	*MicrococcusLuteus* pH 8	Total Positives
1st Quarter	9,80	7,84	5,88	15,69	27,45
(n=51)	(5)	(4)	(3)	(8)	(14)
2nd Quarter	3,92	3,92	0,00	7,84	15,69
(n=51)	(2)	(2)	(0)	(4)	(8)
3rd Quarter	19,61	21,57	11,76	27,45	47,06
(n=51)	(10)	(11)	(6)	(14)	(24)
4th Quarter	5,88	5,88	3,92	11,76	19,61
(n=51)	(3)	(3)	(2)	(6)	(10)
Total positives	9,80	9,80	5,39	15,69	27,45
(n=204)	(20)	(20)	(11)	(32)	(56)

DISCUSSION

The presence of ABs residues in foodstuffs of animal origin is due to a massive, prolonged and uncontrolled use of antibiotics in intensive farming. It also results from non-respect of withdrawal periods (time between the last antibiotic treatment and slaughter of treated animals) that can be justified by economic considerations. [5,9,16].

Several screening tests of ABs residues in foodstuffs of animal origin have been developed. They are essentially microbiological, immunochemical and chromatographic technics [18]. The choice of the microbiological method of FPT was dictated by financial and material constraints. Indeed, this test is simple, fast, effective and accurate [15,17,19]. It allows the analysis of a large number of samples in a short time and the search of residues belonging to five families of ABs with detection thresholds that are very close to their maximum residue limits (MRL) [2,5,16]. These reasons have made this technique the official method of ABs residues detection in foodstuffs of animal origin in the European Union and a test of choice for many scientific researchers worldwide. However, this method allows qualitative identification only, not quantitative, and it does not cover all families of ABs used in broiler like quinolones [20]. Thus, the obtained results by this method must be confirmed by more sophisticated tests.

In Morocco, the regulation in force establishes the list of ABs authorized for each species and the conditions for their use in livestock farming. However, there is no legislation that establishes maximum limits of veterinary drugs residues and official methods of their research in foodstuffs of animal origin [21]. Meanwhile, since 2011, the National Health Security Office of Foods (Office National de Sécurité Sanitaire des Aliments: ONSSA) has implemented a national control plan of ABs residues [21] but their results were never published. NAIM H. has initiated this kind of studies in 2006. He used a microbiological test (STOP: Swab Test On Premises) and a chromatographic technique (GC/MS) to determine antibiotic residues in meat, kidney and liver of poultry and cattle in Rabat city. In the region of Eastern Morocco, we have initiated a preliminary work with 30 samples collected in Oujda city whose results were published by Jaber H. in 2012.

The obtained results show a very significant difference between rates recorded in summer and those recorded in winter, spring and autumn. In fact, summer records the highest rate of positive samples (47.06%), followed by winter (27.45%) and autumn (19.61%). Spring registered the lowest rate of positive samples (15.69%). These prevalences reflect that ABs consumption is irregular in the year. Summer may be the season that registers a maximum of ABs prescription in animal husbandry. These results confirm those of an earlier exhaustive survey conducted by our team among private vets in the region of Eastern Morocco. This survey revealed that the maximum of ABs use in broilers was recorded during summer [7]. This high use of ABs in summer can be explained by high levels of poultry production during this season due to a high demand for weddings and for returning migrants.

In Iran, 22% of samples were reported positive and containing sulfonamides of which 1% contained aminoglycosides and no sample contained β-lactams, tetracyclines or macrolides [8]. In Algeria, similar work has recorded higher prevalences with 86.20% positive samples including 64.83% containing β-lactam and/or

tetracyclines, 31.03% containing sulfonamides, 11.72% containing aminoglycosides and 37.93% containing macrolides and/or β-lactams [11]. In Senegal, 20% of broiler carcasses were reported positive to the FPT of which 4% containing β-lactam and/or tetracyclines, 3% containing sulfonamides, 2% containing aminoglycosides and 15% containing macrolides and/or β-lactams [22]. In Morocco, the only work that was interested in ABs residues in poultry meat is that of Naim H. in 2006 who has found 4.91% of positive samples using the STOP method.

Research works of the seasonal prevalences of ABs residues in broiler meat are very rare. In Pakistan, Habib AMJAD (2007) found that the contamination levels of poultry meat by quinolones, fluoroquinolones and tetracyclines residues were highest in summer and lowest in winter. However, in Bulgaria, a study recorded no positive sample in summer against 4% during winter [23].

The detection of ABs residues in foodstuffs of animal origin reflects a misuse of ABs in animal husbandry. Their presence hamper international trades of these commodities with the sanitary and phytosanitary rules of the World Trade Organization and expose consumers to toxicological, immunological and microbiological hazards [9].

CONCLUSION

The presence of antibiotics residues in foodstuffs of animal origin results mainly from a massive and irrational use of antibiotics in animal production chains. These residues are endangering the consumer's health and impede international foodstuffs trade supervised by the World Trade Organization standards.

More than a quarter of broiler meat samples, tested as part of this work, have been positive to the Four Plates Test. The maximum of positive samples was recorded during summer. Macrolides and β-lactams were the most founded residues in analysed samples.

These results call for taking drastic measures to preserve public health through the rationalization and strict control of antibiotics in intensive farming, the development of antibiotic alternative products and the establishment of an effective control strategy of antibiotics residues in foodstuffs of animal origin.

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