



## Incidence of Antimicrobial Residues in Meat Using a Broad Spectrum Screening Strategy

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### Authors' contributions

*This work was carried out in collaboration between all authors. Author SC headed the project.*

### Article Information

DOI: 10.9734/EJNFS/2015/13795

Original Research Article

Received 4<sup>th</sup> September 2014

Accepted 6<sup>th</sup> March 2015

Published 6<sup>th</sup> April 2015

## ABSTRACT

**Aims:** The aim of this paper was to assess the incidence of antimicrobial residues in market muscle samples from different animal species (bovine, ovine, poultry and porcine) using a new screening strategy.

**Methodology:** 4849 samples were evaluated with a methodology that combines a broad spectrum microbial test (Explorer) and a specific test for quinolones detection (Equinox). Supplementary tests were performed to achieve additional information about the nature of antimicrobials in positive samples.

**Results:** In a first step, 355 samples (7.3%) showed a positive result in Explorer and/or Equinox tests. The highest incidence of positive samples was obtained in poultry (9.7%) while the lowest rate was found in porcine samples (3.4%). Half of the positive screening samples (53%) showed also a positive result with supplementary tests indicating that tetracyclines, aminoglycosides sulphonamides and quinolones might be present in these samples. Aminoglycosides were the predominant residues in poultry while tetracyclines were more frequent in bovine and porcine samples. Sulphonamides were the main family of residues found in ovine.

**Conclusion:** Our results suggest that the current strategies used for control of antimicrobial

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residues in muscle could not be adequate enough. In order to protect consumers from antibiotic exposition, it should be advisable to implement more efficient methods for the screening of antibiotic residues in muscle.

*Keywords: Screening; antibiotic; antimicrobial residues; muscle; microbiological test.*

## 1. INTRODUCTION

The use of pharmacological products in livestock –for treatment of animal diseases or prophylaxis purposes –might lead to the presence of antimicrobial residues in muscle and other organs from animals. As a consequence, several potential concerns may occur: Allergic reactions in sensitized individuals, technological problems in fermented products and toxicological effects [1-3]. Nevertheless, most experts consider that the selection of antimicrobial resistant pathogenic bacteria is the main risk derived from the use of antimicrobials in farm animals.

Maximum Residue Limits (MRLs) for veterinary drug residues in different animal tissues and milk were set by the European legislation [4]. Therefore, food of animal origin must be analyzed to ensure that residues do not exceed MRLs. Since antimicrobials do not share a common chemical structure, it could be necessary to use different analytical procedures to detect every family or even each single compound. Thus, in a first step, efficient control of residues would require screening tests, which are expected to be cheap, easy to perform, allow simultaneous analysis of large numbers of samples and give rapid results [5]. Screening tests are qualitative tools that can differentiate inhibitory samples (samples with antimicrobial residues concentration above detection limits of the test) from non-inhibitory samples. Microbial tests are generally used at screening level and they are based on a bacterial growth inhibition produced by residues contained in the samples [6]. A post-screening step (usually microbial multi-plate tests or immunological methods) may be performed after screening test to identify a family of antibiotics. Finally, positive screening samples must be confirmed, generally with chromatographic methods coupled to mass spectrometric detection [7,8].

Microbial agar diffusion tests have been widely used for detection of antibiotic residues in foods from animal origin. Among them, the most traditional methods are multiplate screening tests involving several bacterial strains; these methods were extensively reviewed by Pikkemaat [8].

Although they are relatively easy to perform, they are usually time-consuming, display a poor sensitivity for sulphonamides and could exhibit wide variations in the performance between laboratories [9]. Microbial inhibition tube tests using an indicator of bacterial growth have been also developed for the screening of antimicrobial residues in muscle. Generally, these tests use a medium seeded with *Geobacillus stearothermophilus* and a pH or redox indicator. The interpretation of results is based on the colour change of the medium caused by microbial growth when antibiotics are not present at inhibitory concentrations in the sample. Tube tests have several advantages since they are generally ready to use, easy to perform and can detect a broad range of antimicrobial residues. Furthermore, results can be obtained in a shorter time (< 3 h) and a photometric reading may be applied, avoiding variations due to visual interpretation made by different technicians or performed over different days [10,5].

However, screening tests based on *G. stearothermophilus* are not able to detect quinolones at MRL levels [11,12]. Consequently, these tests should be combined with specific methods able to detect quinolones in order to cover a wider range of antimicrobials at screening step [13].

The objective of the present study was to assess the incidence of antimicrobial residues in market muscle samples from different animal species (bovine, ovine, poultry and porcine). To achieve this goal, a large number of muscle samples were evaluated with a screening strategy that combines a broad spectrum microbial test (Explorer) with a specific test for quinolones detection (Equinox).

## 2. MATERIALS AND METHODS

### 2.1 Screening Tests

#### 2.1.1 Explorer test

Explorer (ZEULAB, Zaragoza, Spain) is a qualitative test kit for the detection of inhibitory substances in raw meat and other matrices

(kidney, liver, feed and eggs). The test is based on the inhibition of microbial growth of *G. stearothermophilus*. Each well contains an agar-based medium spread with the target bacteria and a pH indicator. When the test is incubated at 65°C, spores germinate and cells grow producing acid and changing the medium pH. Variations of pH will cause changes of the medium colour from blue to yellowish. When the sample contains inhibitors concentrations above detection limits (LOD) of the test, microorganisms will not grow and neither colour changes will be observed.

Samples were extracted by heating a piece of lean meat (3±0.5 g) without adipose or conjunctive tissue in a microwave ("defrost" setting for 1–2 min) and clarified by centrifugation (2000 g for 3 min). Sample meat fluid was added (0.1 ml) to each well with a micropipette. The wells were pre-incubated at room temperature for 20 min to allow the sample to diffuse through the well. Afterwards, the sample was eliminated by washing the wells with distilled water. Finally, the wells were sealed with an adhesive film and incubated at 65±1°C. The endpoint of the assay was reached when the negative control sample (antibiotic-free meat fluid) turned yellow. An objective interpretation of the results was made by performing photometric measurements. The plate was ready to be read when the result for the negative control sample (difference of absorbance at 590 nm and 650 nm) was between 0.15 and 0.25 OD (optical density units). A sample was declared positive when:

$$SA_{590\text{ nm}} - SA_{650\text{ nm}} \geq NA_{590\text{ nm}} - NA_{650\text{ nm}} + 0.15$$

where

NA is the negative control absorbance and SA the sample absorbance.

The performance characteristics of Explorer test for detection of antibiotic residues in muscle from different animal species have been described previously, including detection capabilities (CC $\beta$ ), specificity, false-positive rate and robustness [10,14].

### **2.1.2 Equinox**

Equinox (ZEULAB, Zaragoza, Spain) is a specific kit test for quinolones detection in several food matrices. The test is based on the inhibition of microbial growth of *Escherichia coli* ATCC

11303. The kit includes ampoules with a standardized number of freeze-dried bacteria and ampoules with a specific detection medium containing a redox indicator. During incubation time at 37°C bacterial cells will multiply and modify the redox potential of the medium. As a consequence, a colour change in the medium (from blue to brown/orange) will be observed. Samples containing concentrations of quinolone residues above the Equinox LODs will inhibit the growth of *E. coli* and will prevent the indicator colour change.

The extraction procedure of samples was performed as described previously (2.1.1). Prior to the analysis, the ampoule with *E. coli* was resuspended with the specific detection medium. The assay was carried out in microtiter plates, mixing gently 50 uL of sample or control with 200 uL of reconstituted *E. coli*. The wells were sealed with an adhesive sheet and incubated at 37±1°C.

The endpoint of the assay was reached when the negative control sample (antibiotic-free meat fluid) had turned brown-orange. Equinox results were evaluated by a photometric measurement for an objective interpretation. The assay ended when the result for the negative control sample (difference of absorbance at 590 nm and 650 nm) was between 0.2 and 0.5 OD (optical density units). A sample was declared positive when:

$$SA_{590\text{ nm}} - SA_{650\text{ nm}} \geq NA_{590\text{ nm}} - NA_{650\text{ nm}} + 0.4$$

where

NA is the negative control absorbance and SA the sample absorbance.

An evaluation of the performance of Equinox as a test for detection of quinolone residues in muscle was carried out by Sanz et al. [13].

### **2.1.3 Supplementary screening tests**

To achieve additional information about the chemical nature of antimicrobials contained in screening positive samples, the following supplementary screening tests were performed:

- Test for tetracyclines: Plate with *Bacillus subtilis* (BGA), pH 6 [15].
- Test for aminoglycosides: plate with *B. subtilis* (BGA), pH 8 [15].

- Test for beta-lactams/macrolides: Plate with *Kocuria rhizophila* ATCC 9341, pH 8 [15].
- Test for sulphonamides: the plate seeded with *B. subtilis* (BGA) at pH 7.2 [15] was not suitable for our purpose since it cannot detect sulphonamides in muscle at MRLs [16]. As an alternative, an additional analysis was performed using 4-aminobenzoic acid (PABA, Sigma-Aldrich, Steinheim, Germany): a sample with PABA (50 µg/ml of muscle fluid) and without PABA were analyzed again with Explorer test at the same time. A loss of inhibitory effect was observed in Explorer test when PABA was added to samples spiked with sulphonamides (up to 2000 µg/Kg of sulfadiazine, sulfametoxipiridazine, sulfametazine or sulfathiazole) [17].
- Test for beta-lactams: since plate seeded with *K. rhizophila* ATCC 9341 at pH 8 was not able to discriminate if inhibitory samples contained beta-lactams or macrolides, an alternative was performed to detect specifically beta-lactams. Thus, an additional analysis with Explorer was performed using penicillinase (Sigma-Aldrich, Steinheim, Germany) in a similar way as described previously for sulphonamides: Explorer results were compared when analyzing at the same time a sample without and with penicillinase (100 µg/ml of muscle fluid) [18]. When penicillinase was added to a sample spiked with penicillins (up to 1000 µg/Kg of amoxicillin, bencilpenicillin, cephalixin or ampicillin) a loss of inhibition effect was observed [17].

## 2.2 Evaluation of LODs

Antimicrobial standards of known purity with certificates of analysis were purchased from Sigma-Aldrich (Steinheim, Germany). Antibiotic and sulphonamide stock solutions (1 mg/ml) were prepared and aliquots were kept at -20°C for no more than 2 months. For evaluation of detection limits, intermediate dilutions were obtained in water immediately before every assay and final testing concentrations were prepared in negative bovine muscle fluid.

LODs of Explorer and Equinox for different antimicrobials were published previously [10,13]. Sensitivity data from both studies are compiled in Table 1. Moreover, additional sensitivity data for

some antimicrobials were determined in the present study.

## 2.3 Samples

A total of 4849 muscle samples were analyzed, comprising different animal species: bovine (1302), ovine (1283), poultry (1280) and porcine (984). Bovine, ovine and porcine samples were taken from thoracic diaphragm at different slaughterhouses in the north of Spain while poultry ones (chicken thighs) were purchased from different supermarkets. Samples were individually identified with a code related to animal specie and origin. Muscle samples were transported under refrigeration and screening tests (Explorer and Equinox) were performed in the laboratory upon arrival. Then, samples were frozen and kept at -20°C.

## 2.4 Sample Extraction

The extraction protocol recommended by the manufacturer of the tests was applied to the samples (see 2.1.1 and 2.1.2.). Briefly, meat fluid was extracted by heating in a microwave and was subsequently clarified by centrifugation [10,13].

## 2.5 Analysis of Samples

The antimicrobial screening strategy performed in this study is summarized in Fig. 1. Muscle samples were evaluated, in a first step, with Explorer and Equinox tests. Positive samples detected with Explorer were confirmed by a new assay with this test. If the inhibitory effect was observed also in the second analysis, the sample was further analyzed with specific screening tests for tetracyclines, aminoglycosides, beta-lactams, macrolides and sulphonamides. Besides, positive samples detected by Equinox were tested again with the same test to confirm the results. No more tests were performed to positive samples in first and second analysis, since Equinox is a highly specific test for quinolones.

## 3. RESULTS AND DISCUSSION

### 3.1 Results

#### 3.1.1 Screening of meat samples

In the first step of the study, 4849 samples were analyzed for antibiotic residues with Explorer and

Equinox. As described previously, every positive result in Explorer or Equinox was confirmed with a second analysis in the same test. Only samples that showed inhibitory effect in both assays would be considered as positives. Every sample that showed a positive result at the first screening step remained as positive in a second analysis with the same test. As it is summarized in Table 2, 355 samples (7.3%) were identified as positive by Explorer and/or Equinox test. The highest incidence of positive samples was obtained in poultry (9.7%) while the lowest rate was found in porcine samples (3.4%).

**Table 1. LODs ( $\mu\text{g/Kg}$ ) of Equinox and Explorer for several antibiotics and sulphonamides in bovine muscle (from [10, 13])**

LOD ( $\mu\text{g/kg}$ )	Equinox	Explorer	UE-MRL
Doxycycline	$\leq 5000$	200	100
Tetracycline	n.e.	500*	100
Oxytetracycline	n.e.	700*	100
Penicillin G	$> 5000$	5*	50
Amoxicillin	n.e.	10	50
Cefalexin	n.e.	$> 500$	200
Gentamycin	n.e.	400*	50
Neomycin	$\leq 10000$	300*	500
Tylosin	$> 10000$	100	100
Erythromycin	n.e.	200*	200
Enrofloxacin	100	n.e.	100 <sup>a</sup>
Norfloracin	200	n.e.	
Sarafloxacin	100	n.e.	
Marbofloxacin	100	n.e.	150
Ciprofloxacin	25	n.e.	100 <sup>a</sup>
Danofloxacin	200	n.e.	200
Difloxacin	300	n.e.	400
Flumequine	2000	n.e.	200
Sulfathiazole	$> 10000$	200	100
Sulfamethoxy-pyridazine	n.e.	300*	100
Sulfadiazine	n.e.	200*	100

LODs: Limits of detection, UE-MRL: maximum residue limits EEC (2377/90), <sup>a</sup>: sum of enrofloxacin and ciprofloxacin, n.e.: not evaluated, \*: data provided by manufacturer

**Table 2. Screening results of meat samples with Explorer and Equinox**

	Number of samples	Results	
		Negative (%)	Positive (%)
Bovine	1302	1210 (92.9)	92 (7.1)
Ovine	1283	1177 (91.7)	106 (8.3)
Poultry	1280	1156 (90.3)	124 (9.7)
Porcine	984	951 (96.6)	33 (3.4)
Total (%)	4849	4494 (92.7)	355 (7.3)

### 3.1.2 Supplementary screening test (classification of samples)

Supplementary analyses were performed to Explorer positive samples to confirm results and obtain additional information about the nature of these samples. Results obtained with these tests are summarized in Table 3. Half of the positive screening samples (3.9% of total analyzed samples) showed also a positive result with at least one of the supplementary tests. According to Table 3, these samples might contain residues of tetracyclines (22.3%), aminoglycosides (23.9%), sulphonamides (21.8%), and quinolones (15.4%). Furthermore, results suggest that aminoglycosides and tetracyclines could be combined in 31 samples (16.5%) since an inhibition was observed in two different specific tests at the same time. None of the evaluated samples was found positive at plate seeded with *K. rhizophila* or Explorer with penicillinase. In such a way, a low probability of finding residues of macrolides and beta-lactams would be expected in positive screening samples.

A different pattern was observed when comparing the results from different animal species. Thus, aminoglycosides might be present in 66% of poultry samples but were less common in other species (23-41%). By contrast, data suggest that tetracyclines might be the most frequent residues in bovine and porcine samples while sulphonamide residues were the main family of residues expected in ovine. Quinolone residues might be found in every species (16-26%) except for bovine samples.

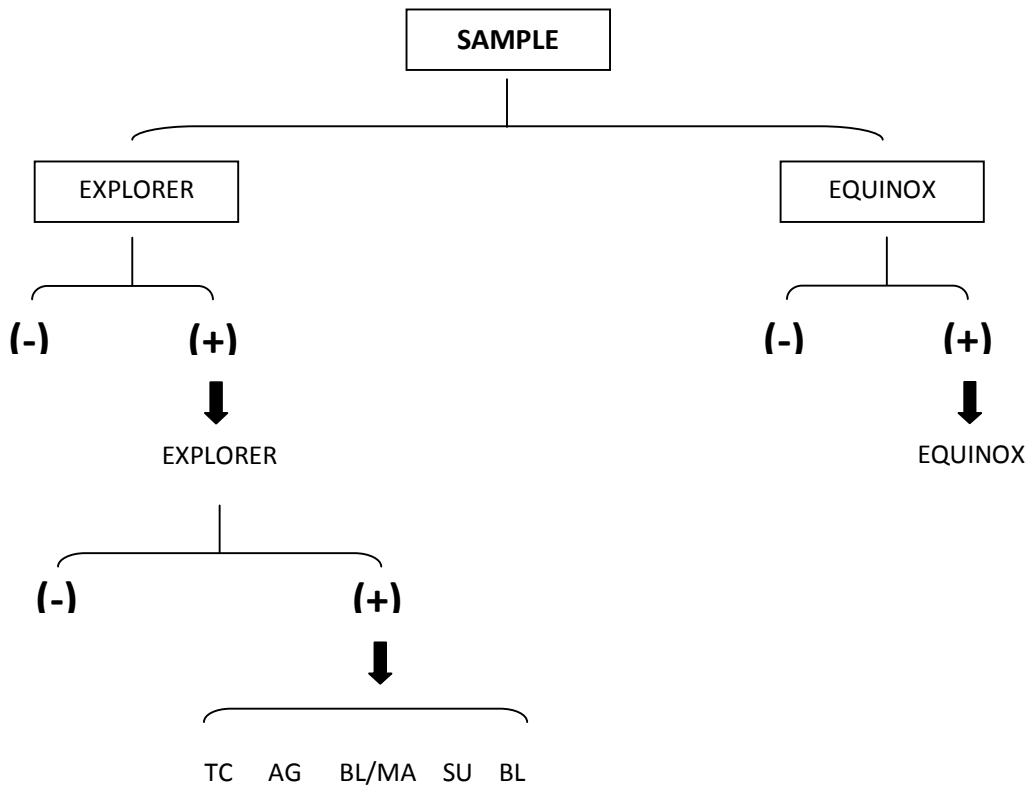
### 3.2 Discussion

The screening strategy used in this work aimed to detect a large range of antimicrobials in muscle samples. The screening system combined 2 microbial methods: A broad spectrum test (Explorer) and a specific test for quinolones (Equinox). Gaudin et al. [10] reported that Explorer was able to detect compounds belonging to different antimicrobial families (penicillins, cephalosporins, tetracyclines, sulphonamides and macrolides) in muscle samples from different species (bovine, porcine, ovine and poultry). These authors observed that detection capabilities were around MRL levels for tested antimicrobials and concluded that Explorer might be used as a wide spectrum screening test for antimicrobials in muscle samples. However, a lack of sensitivity to quinolones is expected in

microbiological tests based on *G. stearotherophilus* [11,12]. Equinox test was found to detect several quinolones (enrofloxacin, ciprofloxacin, marbofloxacin, norfloxacin, sarafloxacin, danofloxacin, difloxacin) in muscle samples of different animal species. The test showed an adequate sensitivity and LODs reported for most of evaluated quinolones were at or below the established MRLs. Furthermore, Equinox exhibited a much lower sensitivity for other groups of antimicrobials since concentrations of doxycycline, tylosin, neomycin, penicillin G and sulfathiazole up to ten times

higher than MRL levels were required to inhibit the test. Therefore, it was concluded that Equinox was a suitable and very specific tool for the screening of quinolone residues in muscle samples [13].

Complementary sensitivity patterns reported for Explorer and Equinox suggest that the screening system used in this work could be an appropriate choice for detection of a wide spectrum of antimicrobial residues in muscle. Thus, a positive result in Equinox may be associated to a sample that contains quinolone residues above Equinox



**Fig. 1. Antimicrobial screening strategy**

TC: test for tetracyclines, AG: test for aminoglycosides, BL/ML: test for beta-lactams/macrolides, SU: test for sulphonamides, BL: test for beta-lactams

**Table 3. Classification of samples: Supplementary screening test results**

	TC	AG	TC + AG	SU	Q	BL/MA	BL
Bovine	24	6	12	2	0	0	0
Ovine	8	4	12	33	11	0	0
Poultry	2	31	4	2	14	0	0
Porcine	8	4	3	4	4	0	0
Total	42	45	31	41	29	0	0
%	22,3	23,9	16,5	21,8	15,4	0	0

Results are expressed as the number of samples giving a positive result with each supplementary test, TC: test for tetracyclines, AG: test for aminoglycosides, SU: test for sulphonamides, Q: test for quinolones, BL/ML: test for beta-lactams/macrolides, BL: test for beta-lactams

LODs while a positive result in Explorer would indicate the presence in the sample of residues from other antimicrobial groups. A positive result observed simultaneously with both tests might be caused by a very high concentration of non-quinolone antimicrobial residues or by the presence of quinolones together with other antimicrobials [13].

It is generally agreed that samples should be screened in a first step with easy, quick and inexpensive methods. These methods are expected to distinguish positive samples (containing levels of antibiotic residues above MRL levels) from the great majority of negative samples [19]. Therefore, screening tests should have the capability for a high sample throughput. Laborious microbial methods involving several plates and bacterial strains could limit their applicability to analyze simultaneously a large number of samples. In this way, the use of Explorer and Equinox tests would allow to analyze a considerable number of muscle samples with a reasonable effort. Moreover, both tests provide a remarkable advantage over other screening tests since the results can be interpreted in a objective way through a photometric reading. Thus, variations in visual reading made by different technicians or performed over different days are avoided, ensuring computer recording and traceability of assays. In addition to that, these ready-to-use tests allow prolonged storage, enhancing operational flexibility in analysis laboratories.

Beta-lactams, tetracyclines, aminoglycosides, macrolides, sulphonamides and quinolones are considered the most widespread antibacterial drugs used in food producing animals [6]. Sensitivity patterns of supplementary specific tests applied to positive screening samples in this work would cover the most commonly used antibacterial drugs in food producing animals and could allow to display a first classification of positive samples according to the family of residues.

The incidence of positive samples observed in our study after analyzing 4849 muscle samples with Explorer and Equinox tests was 7.3%. Nevertheless, Explorer sensitivity data provided by the manufacturer could imply that Explorer could not be sensitive enough to detect tetracyclines at MRL levels (Table 1). Consequently, the real incidence rate of samples containing antimicrobial residues above MRL

levels might be even higher than figures included in this work.

Above half of the positive samples showed a positive result with at least one of supplementary screening tests. Several reasons could explain disagreements between results of screening and additional tests. For example, higher LODs in supplementary screening tests than in Explorer for certain antimicrobials could justify this fact. Thus, the presence of macrolides or aminoglycosides residues at concentrations able to inhibit Explorer could not be sufficient to get a positive result in plates seeded with *K. rhizophila* and *B. subtilis* at pH 8 [10,20,16]. Moreover, degradation of antimicrobial residues in samples along freezing storage could be also involved in obtained results [21,22].

Several studies performed in different countries to evaluate the incidence of antimicrobial residues in meat show substantial variability. Regional animal husbandry, antimicrobial treatment patterns and slaughter practices could be involved. Moreover, differences in the number of tested samples or used methodologies could make difficult an evaluation of published data. As a consequence, comparisons between studies should be taken carefully. Pikemaat et al. [23] observed a 10.8% incidence rate of inhibitory samples after analyzing 591 slaughter animals samples from different species with several microbial tests (Nouws antibiotic test, STAR protocol and Premitest). Furthermore, a higher incidence rate was reported in other study [24] when evaluating 351 muscle samples with a test based on *B. subtilis*. On the other hand, results published by Okerman et al. [19] were more in accordance with our data. Beta-lactams, tetracyclines and fluoroquinolones were detected in 8.3% of chicken samples with a combination of three plates seeded with different bacterial strains.

Results from supplementary screening assays (Table 3) indicate that up to 39% of inhibitory samples may contain tetracycline residues. Our data are in agreement with results reported by Darwish et al. [25] in animal-derived foods and by Salama et al. [26] in chicken samples. These authors observed that 38-42% of samples contained tetracyclines residues. Moreover, high levels of tetracycline residues were found in other studies in different animal species [19,27-29]. However, lower tetracycline rates (4.3-18%) were observed by other authors [23,30].

Occurrence of beta lactams reported in published works showed considerable variations. While Okerman et al. [19] found beta-lactam antibiotics in less than 1% of chicken samples when performing confirmation methods other authors reported that 18% of animal-derived foods in Africa contained beta-lactam residues [25]. Results obtained in our work (Table 3) suggest that no beta lactam residues were present in analyzed samples.

Er et al. [31] and Pena et al. [32] observed a high incidence of residual quinolones in muscle samples (44-58%). On the contrary, quinolone residues were identified much less frequently by other authors [33-36]. Furthermore, Okerman et al. [19] found no quinolones in chicken samples using a multiplate microbial test. Overall occurrence of quinolone residues in different animal species observed in our study might range from 16 to 26%.

Published data on occurrence of antimicrobial residues in muscle from different countries suggest that strategies used for control of residues could not be adequate enough. As a consequence, muscle samples with antibiotic residues above MRL levels could reach consumers and toxicological effects or allergic reactions might occur [1,2]. Moreover, a high incidence of samples containing antimicrobial residues may be linked to an overuse or inappropriate use of antimicrobial drugs in food-producing animals [37]. Hence, the antimicrobial resistance problem might be driven by killing susceptible strains and selecting those that are resistant [38,39]. In this way, the implement of more suitable strategies in antimicrobial residues monitoring plans could help to minimize the incidence of residues concentrations above MRL levels in foods and might have a positive effect on controlling the development and spread of antimicrobial resistance mechanisms.

#### 4. CONCLUSION

Monitoring large numbers of slaughter animals for the presence of antimicrobial residues requires cheap and easy qualitative screening methods. In practice this screening step is primarily performed using microbiological screening tests, because of their high cost-effectiveness compared to physical-chemical methods. The proposed screening strategy (combination of Explorer and Equinox tests) appears to be a useful tool since it would enable a broad screening of antimicrobials in muscle

samples. The analysis of a large number of muscle samples from different animal species have been performed using the proposed strategy. Positive screening results were observed in 7.3% of analyzed samples. Tetracyclines, sulphonamides and aminoglycosides were the most frequent residues found in inhibitory samples.

#### ACKNOWLEDGEMENTS

This investigation was carried out as part of the project PM025/2007 supported by financial assistance from the Diputación General de Aragón (DGA, Spain).

#### COMPETING INTERESTS

Authors have declared that no competing interests exist.

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