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Performance of a New Microbial Test for Quinolone Residues in Muscle

David Sanz · Luis Mata · Santiago Condón · María Ángeles Sanz · Pedro Razquin

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Abstract Concerns regarding the presence of drug residues in foods include allergic reactions, toxicity, technological problems in fermented products and the development of antibiotic resistance in human pathogens. The analysis of antimicrobial residues in foods is generally carried out, in a first step, through microbiological screening tests. These tests commonly use *Geobacillus stearothermophilus* as target specie but show a low ability to detect quinolones. The goal of our study was to evaluate the performance of a new microbiological test (Equinox) for detection of quinolone residues in muscle. The kit contains an ampoule with a standardized number of freeze-dried *Escherichia coli* and must be diluted with a specific detection medium containing a redox indicator. Microbial growth will modify the redox potential of the medium being observed through a colour change (from blue to brown/orange). Equinox limits of detection for most of tested quinolones (enrofloxacin, norfloxacin, sarafloxacin, marbofloxacin, ciprofloxacin, danofloxacin and difloxacin) were below or around maximum residue limit (MRL) UE and CCβ values obtained corresponded with the determined sensitivities. In contrast, flumequine could not be detected at MRL UE levels. Moreover, Equinox displayed a low sensitivity to other antimicrobials. Sensitivity data obtained in vitro were consistent when testing incurred muscle samples. Matrix constituents, test batch and animal species did not affect the performance of the test. Equinox could be easily automated enabling a large numbers of simultaneous analysis, and a photometric reading can be applied for a precise interpretation. The results obtained in this study prove that Equinox is a useful tool when screening for quinolone residues or can be combined with other methods for screening of unknown samples.

Keywords Antibiotic Residues · Quinolones · Screening Method · Inhibition Test · Equinox · Muscle

D. Sanz · L. Mata · P. Razquin
ZEU-Inmunotec S.L.,
C/Bari, 25 Duplicado,
50197 Zaragoza, Spain

S. Condón
Facultad de Veterinaria, Universidad de Zaragoza,
C/Miguel Servet, 177,
50013 Zaragoza, Spain

M. Á. Sanz
Centro de Investigación y Tecnología Agroalimentaria de Aragón,
Avda. Montañana, 930,
50059 Zaragoza, Spain

D. Sanz (✉)
Polígono PLAZA,
C/Bari, 25 Duplicado,
50197 Zaragoza, Spain
e-mail: dsanz@zeulab.com

Introduction

The use of antimicrobials such as antibiotics and sulphonamides is a common practice in livestock. The administration of these drugs may lead to the presence of violative levels of antimicrobial residues in edible products from animals (Anonymous 1990).

Several concerns regarding the presence of drug residues in foods are technological problems in fermented products, toxicity and potential allergic reactions in sensitized individuals (Mäyra-Mäkinen 1995; Cerny 1996; Demoly and Romano 2005). However, the selection of resistant bacteria in farm animals and the subsequent transfer of resistance genes is generally considered as the main risk

derived from the use of antimicrobials in farm animals (Levy 1998; EMEA 1999; WHO 2001). Thus, there is a requirement for rapid, simple and cost-effective tests to screen food of animal origin for the presence of antimicrobial drug residues.

Since antimicrobials do not share a common chemical structure, different analytical procedures are needed to detect every family or even each single compound. Therefore, in a first level, the control of antimicrobials in foods is usually performed by using screening tests—generally, microbiological tests—which should distinguish samples that contain or may contain illegal levels of antimicrobial residues from samples not containing such residues. These tests are relatively cheap and easy to perform and they have a broad spectrum of sensitivity. Microbial screening tests are based on a bacterial growth inhibition produced by concentrations of residues above the detection limit of the test. Most of these tests use *Geobacillus stearothermophilus* as an indicator strain. However, screening tests employing this microorganism are not able to detect quinolones at violative levels in foods because of their poor sensitivity to these antimicrobial compounds (Cullor 1992; Suhren and Heeschen 1996). Consequently, additional testing is required to ensure that the whole antibiotic spectrum is adequately covered.

Quinolones are a group of synthetic antibiotics with both human and veterinary medical uses. These antibacterials are widely used in livestock and fish farm industries (Fàbrega et al. 2008). Quinolones inhibit DNA synthesis by promoting cleavage of bacterial DNA in the DNA-enzyme complexes of DNA gyrase and type IV topoisomerase, resulting in rapid bacterial death (Hawkey 2003; Hoshino et al. 1994; Hooper and Wolfson 1993).

Analytical methods described in the literature for the analysis of quinolones in foods include instrumental techniques (Hernández-Arteseros et al. 1998; Rose et al. 1998; Roybal et al. 2002), rapid immunological techniques (Tittlemier et al. 2008; Scortichini et al. 2009) and microbial methods (Currie et al. 1998; Pikkemaat et al. 2007; Fuselier et al. 2000; Calderón et al. 1996; Myllyniemi et al. 2001; Cantwell and O'keeffe 2006; Gaudin et al. 2009).

Instrumental and immunological techniques provide information on the identities and concentrations of quinolones residues in foods. Nevertheless, these techniques are usually expensive, technically complicated and time-consuming. Thus, they are not suitable when screening a large numbers of samples and microbial multiplate screening methods are widely used for this purpose. These microbial methods usually include a plate seeded with *Escherichia coli* able to detect quinolones residues in foods. Although these tests are simple they are usually time-consuming and could exhibit wide variations in the performance between laboratories (Stead et al. 2004)

A new kit test for quinolones detection in several food matrices (Equinox) recently became available. The kit contains an ampoule with a standardized number of freeze-dried *E. coli* ATCC 11303. Prior to the analysis, the ampoule must be diluted with a specific detection medium containing a redox indicator. The assay is carried out in microwells: bacterial cells will multiply throughout incubation time (37°C) in absence of quinolone residues above the detection limit of the test. This microbial growth will modify the redox potential of the medium and can be observed through a colour change in the medium (from blue to brown/orange). A photometric reading can also be applied for a more accurate interpretation of results. On the other hand, concentrations of residues able to inhibit the growth of *E. coli* will prevent the indicator changing to brown/orange.

The goal of our study was to evaluate the performance of a Equinox test for detection of quinolone residues in muscle.

Materials and Methods

Antibiotic Standards

For preparation of antibiotic and sulphonamide stock solutions, drug standards of known purity with certificates of analysis were purchased from Sigma-Aldrich (Steinheim, Germany) except enrofloxacin, ciprofloxacin (Fluka, Buchs, Switzerland) and sarafloxacin, marbofloxacin, danofloxacin and difloxacin (Riedel-de Haën, Seelze, Germany). Stock solutions were prepared at a concentration of 1 mg ml^{-1} using distilled water (doxycycline, tylosin, neomycin, penicillin G, sulfathiazole), acetic acid 5% (enrofloxacin), sodium hydroxide 1 M (ciprofloxacin, marbofloxacin, danofloxacin, difloxacin and flumequine), sodium hydroxide 0.5 M (sarafloxacin) and glacial acetic acid (norfloxacin). These stock solutions were kept at −20°C for a maximum of 2 months and diluted in distilled water to concentrations suitable for preparation of the spiked samples.

Sample Preparation

Meat samples (bovine/ovine/porcine) were purchased from retail outlets in Zaragoza (Spain) and were tested with a combination of two inhibition tests: STAR protocol (Fuselier et al. 2000) and Explorer kit (ZEU-Inmunotec, Zaragoza, Spain). All samples were inhibitor-negative.

Blank tissue fluid was obtained from fore rib muscle (bovine), leg (ovine) and loin (porcine). A piece of muscle (2–3 g) was cut and placed in a heat-resistant plastic tube and a heating was carried out in a water bath at 100°C for 3 min. Blank tissue fluid was collected and clarified by centrifugation (2,000×*g* for 3 min) and it was kept at −20°C for a maximum of 1 month. Most assays were performed

using bovine tissue fluid while ovine and porcine were utilized for ruggedness testing.

Test Procedure

Equinox test includes vials containing freeze-dried *E. coli*, vials with Q medium (detection medium with a redox indicator) and plastic microwells. The freeze-dried *E. coli* must be reconstituted with the medium.

The assay was carried on microwells, mixing gently 50 uL of sample or control with 200 uL of reconstituted *E. coli*. Non-spiked tissue fluid was used as blank sample. The wells were covered with foil and incubated at 37±1 °C until blank samples had turned brown-orange. At that time (approximately 18–20 h) the wells were examined and a photometric reading was performed.

Readings and Interpretation of the Equinox Results

Equinox results were evaluated both with a visual reading and a photometric measurement. When a visual reading was applied, the wells were held at eye level: blue wells were considered as positive results while brown or orange ones were read as negative ones.

For a photometric reading, the wells must be read at two wavelengths (590 and 650 nm). The results were interpreted as the difference between the values of the two readings. An example of interpretation of quantitative results (optical density) is presented in Fig. 1. The assay ended when the difference of absorbance of blank sample is between 0.5 and 0.2. Cut-off point was set adding 0.4 units to the blank absorbance values. Thus, samples with a difference of absorbance equal or above those obtained for the blank samples plus 0.4 will be positive.

$$\text{Positive} \quad S_{590\text{nm}} - S_{650\text{nm}} \geq (B_{590\text{nm}} - B_{650\text{nm}}) + 0.4$$
$$\text{Negative} \quad S_{590\text{nm}} - S_{650\text{nm}} (B_{590\text{nm}} - B_{650\text{nm}}) + 0.4$$

S sample absorbance
B blank absorbance

Determination of Limits of Detection

Limits of detection (LODs) for ten different antimicrobials belonging to different families were determined: quinolones (enrofloxacin, norfloxacin, sarafloxacin, marbofloxacin, ciprofloxacin, danofloxacin, difloxacin, flumequine), tetracyclines (doxycycline), beta-lactams (penicillin G), macrolides (tylosin), sulphonamides (sulfathiazole) and aminoglicosides (neomycin). For determination of sensitivity, dilutions were obtained in bovine tissue fluid from antibiotic and sulphonamide standards. Spiked samples were prepared from antibiotic and sulphonamide decimal dilutions immediately before every assay.

Concentrations of inhibitors evaluated are shown in Table 1. Samples were tested by ten replicate in every assay. LODs for each antimicrobial was established as the lower concentration that gave rise to a 100% of positive results, according to a photometric evaluation.

Determination of Detection Capability

Solutions of quinolones were prepared in bovine tissue fluid at the limit of sensitivity concentrations determined previously. For each antimicrobial, aliquots of ten different bovine tissue fluid containing the specified concentration were tested.

Examination of Specificity

Ovine tissue fluid was obtained from 20 ovine muscle samples from animals not treated with any antimicrobials for 21 days prior to slaughter. These blank tissue samples were tested in Equinox.

Ruggedness Testing—Effect of Species and Test Batch

Solutions of antimicrobials at detection capability (CCβ) concentrations were prepared in bovine, ovine, poultry and porcine tissue fluid for ruggedness testing. To evaluate the effect of species, Equinox test was performed with

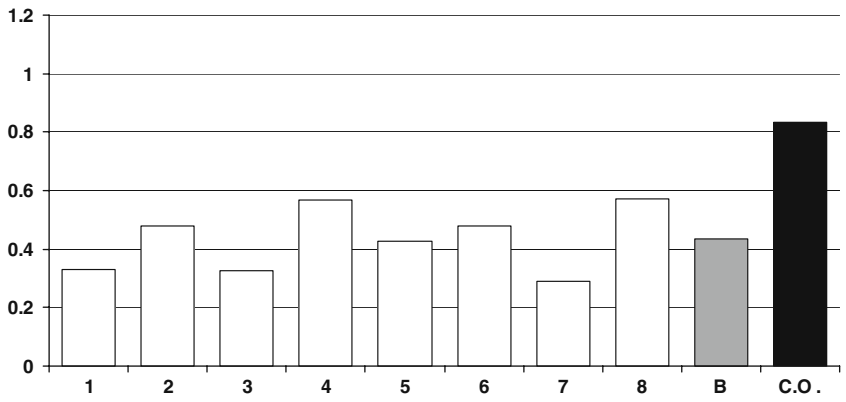

Fig. 1 Example of interpretation of photometric reading. On every assay, a blank sample (*B*) was analysed and the cut-off point (*C. O.*) was set adding 0.4 units to the blank absorbance values (difference of absorbance $B_{590\ \text{nm}}$–$B_{650\ \text{nm}}$). All samples tested in this assay (1–8) were considered negative since their absorbance values ($S_{590\ \text{nm}}$–$S_{650\ \text{nm}}$) were below the cut-off point

Table 1 Concentrations of antimicrobials (diluted in bovine tissue fluid) evaluated in Equinox and MRL UE for each compound in bovine muscle (μg/kg)

Inhibitor	μg/kg	MRL
Enrofloxacin	50, 100, 200	100[a]
Norfloxacin	100, 200, 400	
Ciprofloxacin	25, 50,100	100[a]
Sarafloxacin	50, 100, 200	
Marbofloxacin	50, 100, 150	150
Danofloxacin	50, 100, 200	200
Difloxacin	200, 300, 400	400
Flumequine	500, 1,000, 2,000	200
Doxycycline	100, 1,000, 5,000	100
Tylosin	100, 1,000, 10,000	100
Sulfathiazole	100, 1,000, 10,000	100
Penicillin g	50, 500, 5,000	50
Neomycin	500, 5,000, 10,000	500

[a] Sum of enrofloxacin and ciprofloxacin

antimicrobial solutions (n=5) diluted on tissue fluid from different species. To evaluate the effect of kit batch, antimicrobial solution (n=4) prepared in bovine tissue fluid were tested in Equinox from four different batches.

Quantification of Antimicrobials in Incurred Tissue Samples

Incurred ovine tissue samples were obtained by dosing a group of five 70-day-old Rasa Aragonesa lambs with therapeutic doses of enrofloxacin administered through feed. Another group of five lambs remained untreated as blank samples. In order to achieve samples with high concentration of residues, one animal from every group was slaughtered 1, 2, 7 and 14 days after the start of the treatment, subsequently quartered and *quadriceps femoris* samples were individually packed, freezed (N_2) and stored at −20 °C. With the purpose of evaluate Equinox performance, these ovine tissue samples were analyzed both with Equinox and with a confirmatory method. This confirmatory method consisted of an adaptation of published method by Moats and Romanowski (Moats and Romanowski 1998). In short, 5 g of muscle were extracted with acetonitrile and hexane and the filtrate was evaporated to a final volume of 5 mL. Two millilitres of sample extract were injected in a preparative LC with fraction collector equipment (BioRad). The fractionation was carried out with a Supelcosil C18 (150×4.6 mm, 5 μm, Supelco, USA) using a mobile phase 100% 0.01 M KH2PO4.

The HPLC system used for separation was Agilent Technologies 1100 series equipped with a quaternary solvent delivery system, an auto sampler, a column oven and a diode-array detector. Data were captured with ChemStation software, rev. B.01.03 (Agilent Technologies, USA). Chromatographic separation was accomplished using a Kromasil 100 C18 (20× 4.6 mm, 5 μm) (Teknokroma, Spain) using a mobile phase 0.01 M KH2PO4 pH3.0: acetonitrile (90:10, *v*/*v*).

Results

Determination of LODs

Table 2 shows the limits of detection observed in Equinox for enrofloxacin, norfloxacin, sarafloxacin, marbofloxacin, ciprofloxacin, danofloxacin, difloxacin and flumequine diluted in bovine muscle. Results were obtained both according to a photometric measurement and through a visual examination. Average absorbance value for each sample and variation coefficients are included in the table. Bovine tissue fluid samples containing 100, 200, 100, 100, 25, 200, 400 and 2,000 μg/kg of enrofloxacin, norfloxacin, sarafloxacin, marbofloxacin, ciprofloxacin, danofloxacin, difloxacin and flumequine respectively, were able to inhibit the test Equinox. For other antimicrobials, Equinox displayed a lower sensitivity, as it is shown in Table 3. Doxycycline was detected at a concentration of 5,000 μg/kg while 10,000 μg/kg of neomycin were able to inhibit the test. However, Equinox could not detect tylosin, penicillin G and sulfathiazole at 10,000, 5,000 and 10,000 μg/kg, respectively. Results obtained through visual examination were the same as those from a photometric measurement.

Determination of Detection Capability

To determine the CCβ for the investigated quinolones, 10 different blank bovine muscle samples fortified with enrofloxacin (100 μg/kg), norfloxacin (200), sarafloxacin (100), marbofloxacin (100), ciprofloxacin (25), danofloxacin (200), difloxacin (400) and flumequine (2000) were analysed. All the fortified samples were found to give a positive response; therefore the CCβ corresponds with the determined sensitivities included in Table 2.

Examination of Specificity

Analysis of 20 blank ovine muscle samples gave no false positive results, indicating that matrix constituents do not affect the specificity of Equinox (Fig. 2).

Ruggedness Testing—Effect of Species and Test Batch

The ruggedness of Equinox was examined evaluating the effect of animal species and kit batch on the test results. Table 4 summarizes the effect of animal species on the limits of detection of Equinox test for several quinolones. A positive response was observed for solutions of marbo-

Table 2 Sensitivity (μg/kg) of Equinox for enrofloxacin, norfloxacin, sarafloxacin marbofloxacin, ciprofloxacin, danofloxacin, difloxaicn and flumequine diluted in bovine muscle

	Photometric measurement			Visual interpretation			
	Abs_{AV}	$CV_{\%}$	Result	T1	T2	T3	T4
NC	0.467	5.7	(−)	(−)	(−)	(−)	(−)
Cut-off point	0.867	(+)					
Enrofloxacin							
50	0.160	3.2	(−)	(−)	(−)	(−)	(−)
100	1.441	16.8	(+)	(+)	(+)	(+)	(+)
200	1.443	1.9	(+)	(+)	(+)	(+)	(+)
Norfloxacin							
100	0.193	12.8	(−)	(−)	(−)	(−)	(−)
200	1.456	6.4	(+)	(+)	(+)	(+)	(+)
400	1.492	0.8	(+)	(+)	(+)	(+)	(+)
Sarafloxacin							
50	0.173	16.2	(−)	(−)	(−)	(−)	(−)
100	1.461	5.2	(+)	(+)	(+)	(+)	(+)
200	1.465	1.9	(+)	(+)	(+)	(+)	(+)
Marbofloxacin							
50	0.171	2.9	(−)	(−)	(−)	(−)	(−)
100	1.722	4.2	(+)	(+)	(+)	(+)	(+)
150	1.640	8.0	(+)	(+)	(+)	(+)	(+)
Ciprofloxacin							
25	1.393	20.3	(+)	(+)	(+)	(+)	(+)
50	1.513	2.2	(+)	(+)	(+)	(+)	(+)
100	1.517	1.1	(+)	(+)	(+)	(+)	(+)
Danofloxacin							
50	0.200	9.0	(−)	(−)	(−)	(−)	(−)
100	0.328	5.2	(−)	(−)	(−)	(−)	(−)
200	1.525	2.7	(+)	(+)	(+)	(+)	(+)
Difloxacin							
200	0.177	6.5	(−)	(−)	(−)	(−)	(−)
300	1.201	19.8	(+)	(+)	(+)	(+)	(+)
400	1.591	2.6	(+)	(+)	(+)	(+)	(+)
Flumequine							
500	0.180	19.3	(−)	(−)	(−)	(−)	(−)
1,000	0.452	6.1	(−)	(−)	(−)	(−)	(−)
2,000	1.479	4.1	(+)	(+)	(+)	(+)	(+)

Cut-off point is abs_{AV} NC + 0.4

Abs_{AV} average of abs 590 and 650 nm for every well inoculated with the sample or standard, *$CV_{\%}$* coefficients of variation, *NC* negative control (blank bovine tissue fluid), *T1–T4* visual interpretation result from four different technicians, (+) 100% of positive results, (−) 100% of negative results

floxacin at 100 μg/kg in all cases. However, the sensitivity of Equinox for sarafloxacin in pork and beef was lower than in lamb and chicken while the sensitivity for enrofloxacin was lower in pork. Test batch was not found to affect Equinox performance as it is shown in Table 5. In all cases, the limits of detection of Equinox for enrofloxacin and sarafloxacin were 100 μg/kg.

Quantification of Antimicrobials in Incurred Tissue Samples

Ovine tissue samples containing enrofloxacin and ciprofloxacin were analyzed both with Equinox and with the confirmatory method (Table 6). Samples with 716/104 and 243/140 μg/kg of enrofloxacin/ciprofloxacin, respectively, were found to give a positive response in Equinox test. On the contrary, samples containing 48/15 and 17/0 μg/kg of enrofloxacin/ciprofloxacin were not able to inhibit Equinox.

Discussion

Enrofloxacin, ciprofloxacin, marbofloxacin, danofloxacin and difloxacin were detected in bovine tissue fluid at levels equal to the MRLs (100, 100, 150, 200 and 400 μg/kg). Moreover, ≤100 μg/kg of marbofloxacin and ≤25 μg/kg of ciprofloxacin showed an inhibitory effect on the test. Since

Table 3 Sensitivity (μg/kg) of Equinox for several antibiotics and sulphonamides diluted in bovine muscle

	Photometric measurement			Visual interpretation			
	Abs_{AV}	CV	Result	T1	T2	T3	T4
NC	0.467	5.7	(−)	(−)	(−)	(−)	(−)
Cut-off point	0.867	(+)					
Doxycycline							
100	0.436	11.8	(−)	(−)	(−)	(−)	(−)
1,000	0.436	10.2	(−)	(−)	(−)	(−)	(−)
5,000	1.516	0.27	(+)	(+)	(+)	(+)	(+)
Neomycin							
500	0.418	5.6	(−)	(−)	(−)	(−)	(−)
5,000	0.602	6.0	(−)	(−)	(−)	(−)	(−)
10,000	1.571	1.3	(+)	(+)	(+)	(+)	(+)
Tylosin							
100	0.406	0.4	(−)	(−)	(−)	(−)	(−)
1,000	0.407	4.4	(−)	(−)	(−)	(−)	(−)
10,000	0.352	6.3	(−)	(−)	(−)	(−)	(−)
Penicillin G							
50	0.407	8.9	(−)	(−)	(−)	(−)	(−)
500	0.409	3.1	(−)	(−)	(−)	(−)	(−)
5,000	0.327	5.5	(−)	(−)	(−)	(−)	(−)
Sulfathiazole							
100	0.413	5.4	(−)	(−)	(−)	(−)	(−)
1,000	0.338	8.2	(−)	(−)	(−)	(−)	(−)
10,000	0.348	3.8	(−)	(−)	(−)	(−)	(−)

Cut-off point is Abs_{AV} NC+0.4

Abs_{AV} average of abs 590 and 950 nm for every well inoculated with the sample or standard, *$CV_{\%}$* coefficients of variation, *NC* negative control (blank bovine tissue fluid), *T1–T4* visual interpretation result from four different technicians, (+) 100% of positive results, (−) 100% of negative results

no MRLs for norfloxacin and sarafloxacin have been established for bovine muscle, it is not possible to compare the observed LODs with legal requirements. However, the sensitivity showed by Equinox for norfloxacin and sara-

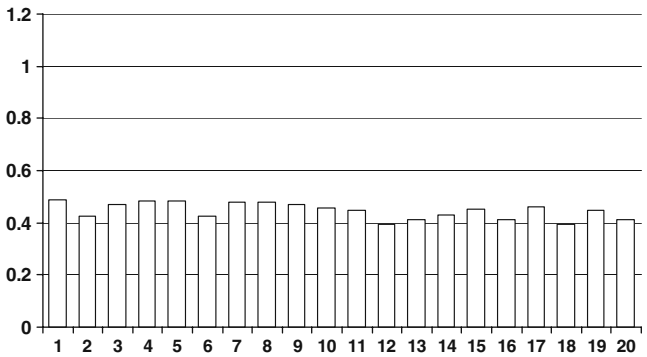

Fig. 2 Effect of matrix constituents on Equinox specificity: analysis of 20 blank ovine muscle samples

Table 4 Effect of animal species on sensitivity of Equinox for several quinolones

	μg/kg	Abs_{AV}	$CV_{\%}$
Marbofloxacin			
Pork	50	0.720	3
	100	1.542	3.3
	150	1.650	1.3
Beef	50	0.171	2.9
	100	1.722	4.2
	150	1.640	8.0
Lamb	50	0.153	10.8
	100	1.511	1.7
	150	1.421	11.2
Chicken	50	0.182	10.2
	100	1.507	1.3
	150	1.390	7.6
Enrofloxacin			
Pork	50	1.582	1.1
	100	1.665	1.9
	200	1.705	1.1
Beef	50	0.160	3.2
	100	1.441	16.8
	200	1.443	1.9
Lamb	50	0.132	5
	100	1.468	7.1
	200	1.424	5.3
Chicken	50	0.121	6.9
	100	1.519	5.9
	200	1.502	3.8
Sarafloxacin			
Pork	50	0.230	7.0
	100	1.606	5.2
	200	1.714	4.5
Beef	50	0.173	16.2
	100	1.461	5.2
	200	1.465	1.9
Lamb	50	0.189	4
	100	0.399	6.7
	200	1.411	8.2
Chicken	50	0.206	7.6
	100	0.192	4.7
	200	1.463	4.1

Negative control: pork (0.461±0.069), beef (0.476±0.024), lamb (0.463±0.067), chicken (0.458±0.023). Cut-off point: pork (0.861), beef (0.876), lamb (0.863), chicken (0.858)

Abs_{AV} average of abs 590 and 950 nm for every well inoculated with the sample or standard, *$CV_{\%}$* coefficients of variation

Table 5 Limits of detection (μg/kg) for enrofloxacin and sarafloxacin in 4 batches of Equinox

	Batch 1		Batch 2		Batch 3		Batch 4	
	Abs_{AV}	$CV_{\%}$	Abs_{AV}	$CV_{\%}$	Abs_{AV}	$CV_{\%}$	Abs_{AV}	$CV_{\%}$
NC	0.467	5.7	0.416	10.3	0.348	10.1	0.409	5.4
Cut-off point	0.867		0.816		0.748		0.809	
Enrofloxacin								
50	0.160	3.2	0.175	5.9	0.188	3.1	0.186	8.2
100	1.441	16.8	1.446	4.7	1.513	2.7	1.341	4
200	1.443	1.9	1.486	3.9	1.490	8.2	1.358	4.2
Sarafloxacin								
50	0.173	16.2	0.210	6	0.198	9.1	0.1965	8.9
100	1.461	5.2	1.429	4.2	1.450	3.9	1.2976	4.3
200	1.465	1.9	1.492	3.6	1.484	4.6	1.3598	3.5

Negative control (blank bovine tissue fluid)

floxacin (200 and 100 μg/kg) in bovine tissue fluid are on the order of established MRLs for other quinolones. For that reason, Equinox sensitivity for norfloxacin and sarafloxacin might be considered acceptable.

Sensitivity studies have been extensively conducted on multiplate assays. A modification of the EC Four Plate Method was developed by Currie et al. (Currie et al. 1998). Minimum inhibitory concentration observed for ciprofloxacin, enrofloxacin and sarafloxacin were 500 μg/kg using standard solutions. A CCβ of ≤25, ≤50, ≤50 and ≤100 μg/kg for enrofloxacin, danofloxacin, difloxacin and flumequine in poultry muscle was reported with an improved microbial screening assay based on the inhibition of *Yersinia ruckeri* NCIMB 13282 (Pikkemaat et al. 2007). Myyniemi et al. (Myllyniemi et al. 2000) evaluated the suitability of a multiplate test based on inhibition of *Bacillus subtilis BGA*. The authors concluded that enrofloxacin and ciprofloxacin residues were not detected at MRL concentration in bovine and porcine muscle samples. Other study by the same author reported that a microbiological six-plate method was able to detect a sum of enrofloxacin and ciprofloxacin at 30 μg/kg (Myllyniemi et al. 2001). STAR protocol was evaluated for antibiotic detection in muscle (Fuselier et al. 2000). Slices of pork meat with 161 μg/kg of enrofloxacin were detected by the plate inoculated with *E. coli* ATCC 11303.

The suitability of *E. coli* for screening of different quinolone residues was also determined using standard solutions in media with different pH values (Okerman et al. 2007). The lowest limits of detection observed for enrofloxacin, ciprofloxacin, marbofloxacin, norfloxacin, sarafloxacin, danofloxacin, difloxacin and flumequine were 0.625, 1.25, 2.5, 5, 5, 1.25, 40 and 5 μg/kg, respectively. A marked influence of medium pH on sensitivity was observed for most of the quinolones evaluated. Moreover, it has been showed previously that inhibition zones observed with meat samples are likely to be much lower (Okerman et al. 1998). In contrast, other studies (Myllyniemi et al. 2000) have concluded that inhibition zones were wider around kidney samples containing antimicrobial residues than around paper discs impregnated with standard solutions. Differences between tissue matrices could explain this disagreement since antimicrobial residue concentration in matrices such as kidney was reported to be higher than concentration in muscle samples. Therefore some authors (Okerman et al. 2007) have concluded that it should be advisable to use kidney rather than muscle samples as the test material for analysis of residues in animals. However, muscular antimicrobial concentration is more relevant for consumer safety than kidney levels. Consequently, it would be preferable to screen for antimicrobial residues in muscle, despite some compounds could be detected at or about the MRL in other tissues such as kidney.

Quinolones have also been detected by other methods such as chromatographic methods, luminescence spectroscopy or immunological tests. Chromatographic or luminescence spectroscopy methods are intended for identification and quantification of residues, and they are not considered a

Table 6 Quantification of enrofloxacin and ciprofloxacin in incurred ovine tissue samples by a confirmation method: comparison with Equinox results

	Enrofloxacin (μg/kg)	Ciprofloxacin (μg/kg)	Equinox
Sample 1	716	104	(+)
Sample 2	243	140	(+)
Sample 3	48	15	(−)
Sample 4	17	0	(−)

first choice for screening a large number of samples since they are specific, more labour-intensive and more expensive than microbial tests (Myllyniemi et al. 2000). Rose at al. (Rose et al. 1998) optimized and validated a multi-residue procedure, with a limit of determination of 10 μg/kg for ciprofloxacin, enrofloxacin, norfloxacin, sarafloxacin and marbofloxacin and 5 μg/kg for danofloxacin. A published terbium-sensitized luminescence method showed a limit of detection for ciprofloxacin and enrofloxacin below 1 μg/L (Hernández-Arteseros et al. 1998). Nevertheless, the sensitivity depended on the matrix composition and the limits of detection were higher when analyzing trout or chicken tissue, ranging from 2.7 to 3.8 μg/kg. Immunological methods are generally used for screening and post-screening, since they are specific for one group of antimicrobials. Enzyme immunoassay kits for the quantitative analysis of quinolones in several matrices have been commercialised by different manufacturers. Generally, these kits have lower limits of detection than microbial methods but cross reactivities between the different quinolones detected are not optimal in most cases (Tittlemier et al. 2008; Scortichini et al. 2009). Antibodies used in ELISA tests for detection of quinolones have shown cross-reactivity values lower than 10% against several evaluated quinolones.

Equinox was found to be less sensitive for other antimicrobial groups. Doxycycline, tylosin, neomycin, penicillin G and sulfathiazole were not detected at concentrations ten times higher than MRLs. Furthermore, Equinox could not detect tylosin, penicillin G and sulfathiazole at concentrations even 100 times above MRLs. In all cases, concentration of antimicrobials able to inhibit Equinox were well above reported limits of detection in broad-spectrum screening tests. However, Gaudin et al observed an inhibitory effect of several cephalosporins on *E. coli* plates (Gaudin et al. 2004). None of the antimicrobials evaluated, except for tested quinolones, was detected in Equinox at MRL concentration. Moreover, results observed when spiking muscle tissue with quinolones or other groups of antimicrobials were in agreement with data from HPLC results.

All the fortified samples with enrofloxacin (100 μg/kg), norfloxacin (200), sarafloxacin (100), marbofloxacin (100), ciprofloxacin (25), danofloxacin (200), difloxacin (400) and flumequine (2000) gave a positive response in Equinox. Therefore, the detection capability for every evaluated quinolone was found to correspond with the sensitivities obtained in Table 2. European legislation requires that the CCβ of screening tests is equal or below the MRL. Thus, Equinox might be approved for official surveys, at least for enrofloxacin, ciprofloxacin, marbofloxacin, danofloxacin and difloxacin. Analysis of 20 blank ovine muscle samples gave no false positive results. These data suggest that matrix constituents do not affect the specificity of Equinox.

Equinox was tested for ruggedness evaluating effect of animal species and test batch according to EU requirements (Anonymous 2000). Animal species were not found to affect Equinox sensitivity for marbofloxacin, since 100 μg/kg gave rise to a positive result in porcine, bovine and ovine muscle. Consequently, concentrations of marbofloxacin at MRL levels would be detected by Equinox in all meat species. In contrast, the sensitivity of Equinox for enrofloxacin was lower in porcine muscle, while sarafloxacin showed higher limits of detection in ovine and chicken. Nevertheless, differences observed could be more influenced by individual factors than by animal species. Supplementary experiments would be necessary to conclude if Equinox sensitivity is affected by animal species. Previous studies reported that performance of other commercial screening tests were not affected by animal species while microbial multiplate methods are considered to be more influenced by kind of samples (Myllyniemi et al. 2000; Okerman et al. 1998). As it is shown in Table 5, sensitivity for enrofloxacin and sarafloxacin in four batches of Equinox was 100 μg/kg. Thus, on the basis of the results it could be concluded that the limit of detection for enrofloxacin and sarafloxacin were not affected by kit batch. Further investigations might be necessary to evaluate the effect of batch kit on sensitivity for other quinolones and even to other antimicrobials.

One of the most critical points when screening for antimicrobial residues is the reading of the results. Positive results are shown in multiplate assays by measuring the inhibition zones observed around samples. Nonetheless, these results could be difficult to interpret since these inhibition zones might be influenced by different parameters, like inoculum concentration, agar depth, temperature of incubation or batch preparation. Moreover, high percentage of false-positive results is often associated with the use of microbial diffusion methods (Pikkemaat et al. 2007). As it is shown in Tables 2 and 3, there were a total correspondence between the results obtained through a photometric measurement and those ones from a visual interpretation. Therefore, a photometric reading might be applied to Equinox, avoiding variations due to visual reading made by different technicians or performed over different days.

It is assumed that multiplate methods using more than one indicator microorganism enable to target a broader range or antimicrobial residues. Therefore, and considering Equinox sensitivity pattern for quinolones and other antimicrobials, the test might be suitable for monitoring meat samples in situations where the presence of residues of quinolones can be expected. In contrast, it should be combined with a broad spectrum test unable to detect quinolones at violative levels in foods (i.e. based on *G. stearothermophilus*) for screening of unknown muscle samples. Performance of Equinox along with other broad spectrum method (i.e. Explorer®, ZEU-INMUNOTEC, Spain or Premitest®, DSM, Netherlands) would enable a virtually complete plan for the screening of antimicrobials in muscle. Thus, a positive result only in

Equinox might suggest that the sample contains quinolones residues above Equinox LODs. An inhibition observed in both tests might be related to a high concentration of antimicrobial residues (able to inhibit even Equinox) or to the presence of one or more quinolones together with one or more antibiotic/sulfonamides.

Conclusion

The Equinox test was found to detect several quinolones (enrofloxacin, ciprofloxacin, marbofloxacin, norfloxacin, sarafloxacin, danofloxacin, difloxacin and flumequine) in the muscles of different animal species (bovine, ovine, porcine and poultry). Except for flumequine, the limits of detection observed for these quinolones were below or on the order of established MRLs. Furthermore, Equinox exhibited a much lower sensitivity for other groups of antimicrobials (doxycycline, tylosin, neomycin, penicillin G, sulfathiazole). However, sensitivity of Equinox to cephalosporins should be investigated. The results obtained in this study prove that Equinox is fit for purpose as a qualitative screening test for quinolone residues (EU commission decision 2002/657/EC). Thus, Equinox test should be considered a useful tool when screening for quinolone residues. However, it should be combined with other methods for screening of unknown samples. Other advantage of the kit is that the results can be obtained through a photometric reading, avoiding variations due to subjective visual interpretation. Moreover, Equinox could be easily automated enabling a simultaneous analysis of large numbers of samples.

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