Collaboration

Validation of the five-plate screening test for the detection of antibiotic residues in food. Estimation of the limit of detection

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Abstract

The five-plate test is a simple and low cost microbiological screening technique widely used in Spain for the detection of antibiotic residues in foods of animal origin.

This work describes the criteria used to validate this technique and the results obtained in different matrices: porcine, bovine, ovine and poultry muscle, bovine kidney, milk, egg and fish, according to criteria established by Commission Decision 2002/657/EC.

The results obtained imply just an estimate of the detection limits of this technique, given that a precise estimation of the detection limits would be only allowed by the use of whole tissue samples containing antibiotics, as in the case of this technique. In addition, the estimate was made using a very high safety margin since only perfectly visible inhibition zones were considered.

Validation of the antibiotic residue screening technique in accordance with Decision 2002/657/EC, despite some antibiotics being detected at higher levels, has demonstrated that the technique allows for detection of a certain number of antibiotics in various foods at or below the maximum residue level. It is therefore valid for the intended purpose.

Key words

Validation, antibiotic residues, screening, detection limit.

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Introduction

The use of veterinary drugs for the treatment of illnesses of animals intended for human consumption is a widely extended legal practice. These animals should not be slaughtered and their production of food should not be used until a period has elapsed since completing the administration of the drug, in order to allow the residues to be eliminated. For that reason, if the so-called withdrawal period is not respected, residues of these drugs may appear in food.

The main health problem caused by the presence of antibiotic residues in food is their toxicity, particularly allergic reactions such as those caused, for example, by penicillins. Furthermore, these residues have technological consequences as they may interfere in food production processes which use bacterial cultures (cheese, yoghourt, cold meats). In turn, as with the human population, the incorrect use of antibiotics encourages bacterial resistance.

As a consequence of the adverse effects that veterinary drug residues may produce in food, the European Union has introduced Maximum Residue Limits (MRL) in food of animal origin.

Thus, Regulation (EC) No 470/2009 (EU, 2009), lays down a Community procedure establishing the maximum limits for residues of pharmacologically active substances in food of animal origin.

In the European Union the sanitary control of veterinary drugs residues in food comes under the framework established by Directive 96/23/EC (EU, 1996), transposed into the Spanish legal system via Royal Decree 1749/1998 laying down the basis of the National Residue Plan (PNIR in its Spanish acronym) (Real Decreto, 1998). This plan lays down the measures of control applying to certain substances and their residues in live animals and their products intended for human consumption. Its main objective is to detect any illegal treatment and check that veterinary drug residues are below their corresponding MRL.

Screening techniques play an important role in the detection of antibiotic residues as the control covers a large number of samples, antibiotics and different matrices. A screening technique makes it possible to select simply and economically those samples which could contain antibiotic residues.

If the screening technique gives a positive result, more specific physicochemical techniques are required to confirm and quantify the residue.

Many laboratories in Spain use the five-plate test technique for screening antibiotic residues; this is a simple, low-cost microbiological assay method. It is based on the inhibition of the growth of bacterial cultures on a plate to detect antibacterial activity of the residues of antibiotics present in foodstuffs.

The five-plate screening test was initially developed as an antibiotic residue detection method with four plates (Bogaerts and Wolf, 1980) and it has been used extensively throughout Europe. A fifth plate was subsequently added, which improves the detection of the quinolones group thanks to the greater susceptibility of a strain of *Escherichia coli* (Ellerbroek, 1991). This method allows, with different modifications, the detection of antibiotic residues in different foods of animal origin such as muscle, kidney, milk and eggs and in animal feeds. Evaluation studies have been published on the detection limit of similar (Currie et al., 1998) or alternative methods (Gaudin et al., 2010).

The analytical methods used for the analysis of official control samples of residues must be validated in accordance with Decision 2002/657/EC (EU, 2002) on the performance of analytical methods and the interpretation of results for screening and confirmation methods. Decision 2002/657/EC unifies at

a European level the requirements for applicable control measures concerning certain substances and their residues in live animals and their products. It is necessary to assure the quality and comparability of the analytical results of the authorized laboratories for the official control of residues; to do this, quality assurance systems must be applied and, specifically, methods validated in accordance with common operational procedures and criteria.

This paper presents the procedure followed in the Spanish National Reference Laboratory for antibiotic residues in food for the validation of this technique, the criteria used and the results obtained.

Validation design

The validation requires a study of the parameters which may influence the result. The ultimate objective is to find out how the method performs and set the criteria it should meet in order to reach valid results concerning the overall objective of its application.

In the validation of the five-plate screening test for antibiotic residues, we have taken the following steps:

1. Definition of the purpose of the method. In this case, the purpose of the method is to detect the largest possible number of antibiotic residues at or below the maximum residue limits (MRL). This only attempts to detect antibiotics which have an MRL as the detection of antibiotics whose use is prohibited (for example, chloramphenicol or nitrofurans) requires a greater sensitivity and specificity than that provided by microbiological techniques.
2. Determine method characteristics. In accordance with Decision 2002/657/EC the study parameters in a qualitative screening technique are the detection limit (CCß), the specificity/selectivity and the ruggedness/applicability/stability.
3. Establish requirements for each parameter:
   - Detection limit: In accordance with Decision 2002/657/EC qualitative screening techniques must have a percentage of false negative results below 5% (ß error) at the level of interest. This means that the detection limit must be equal to or below the MRLs established for each antibiotic with a percentage of false negative results below 5%.
   - Specificity/Selectivity: According to Decision 2002/657/EC, specificity is the capacity of a method to distinguish between the analyte being measured and other substances. In this case, the technique has a very wide detection range so that the requirement is that there should be no systematic interferences from the matrix itself and, if there are, there should be systems to prevent these interferences.
   - Applicability/robustness/stability: Simple, economic and easily applied techniques with good reproducibility are required. In addition, the technique must be robust enough to ensure it is not altered by variations occurring in the time or temperature of incubation, the analysts applying it, etc.
4. Supplying evidence to show that the method meets requirements:
   - Detection limit: There are several possible sources of information such as the participation in intercomparison tests which have used samples from animals treated with antibiotics, the confirmation and quantification of residues from samples testing positive in the screening

technique and the performance of specific trials. In this case, we have opted for the latter system as the number of intercomparison tests using whole tissues is very limited and few samples have tested positive and they do not cover the necessary range of antibiotics and concentrations.

- Specificity/Selectivity: This has to study whether each of the matrices whose analysis can be performed with this technique causes systematic inhibitions of the bacterial growth in the plates used. This technique is not capable of identifying antibiotics within the same family; it only aims to detect the presence of antibiotic residues. The specificity of the technique increases by eliminating the natural inhibitions which might be present naturally in foods.
- Applicability/robustness/stability: A critical assessment of the characteristics of the technique is required, studying the possible variability of the results depending on parameters such as the volume of medium per plate, incubation time, etc.

5. Evaluation and preparation of the report. When the different parameters have been studied, an evaluation of the results of the validation should be performed to conclude whether the analytical method is valid or not for its intended use.

In this study we evaluate the results obtained in the validation of the five-plate screening test for antibiotic residues regarding its detection limit and specificity.

Materials and methods

1. Antibiotic patterns

Amoxicillin Sigma A8523, Ampicillin Sigma A9518, Apramycin Sigma A2024, Bacitracin Sigma B0125, Cefalonium Fluka 32904, Cefazolin Fluka 22127, Cefoperazone Sigma C4292, Cefquinome Fluka 32472, Ceftiofur Vetranal 34001, Cephalexin Sigma C4895, Cephapirin Fluka 43989, Chlortetracycline Sigma C4881, Cloxacillin Sigma C9393, Colistin Sigma C4461, Danofloxacin Vetranal 33700, Dicloxacillin Sigma D 9016, Difloxacin Vetranal 33984, Dihydrostreptomycin Sigma D7253, Doxycycline Sigma D9891, Enrofloxacin Fluka 17849, Erythromycin Sigma E6376, Florfenicol Sigma F1427, Flumequine Sigma F7016, Gentamicin Sigma G3632, kanamycin Sigma K4000, Lincomycin Sigma L6004, Marbofloxacin Vetranal 34039, Monensin Sigma M5273, Nafcillin Sigma N3269, Neomycin Sigma N1876, Novobiocin Sigma N1628, Oxacillin Sigma O1002, Oxolinic Acid O0877 Oxytetracycline Sigma O5875, Paromomycin Sigma P9297, Penicillin G PENNA, Penicillin V Vetranal 46616, Sarafloxacin Vetranal 33497, Spectinomycin Sigma S9007, Spiramycin Vetranal 46745, Streptomycin Sigma S6501, Sulfachloropyridazine Sigma S9882, Sulfadiazine Sigma S8626, Sulfadimethoxine Sigma S7007, Sulfamethazie Sigma S6256, Sulfamethoxazole Sigma S7507, Sulfamethoxypyridazine Sigma S7257, Sulfapyridine Sigma S6252, Sulfaquinoxaline S7382, Sulfathiazole Sigma S0127, Tetracycline Sigma T3383, Thiamphenicol Sigma T0261, Tiamulin Vetranal 46959, Tilmicosin 33864, Trimethoprim Sigma T7883, Tylosin Sigma T6134, and Valnemulin Fluka 32971.

2. Reagents

Sterile distilled water, 0.1N NaOH from Merck 1.06498 sodium hydroxide, 5% acetic acid from Merck 1.00062 96% acetic acid and Merck 1.06007 methanol.

3. Test plate preparation

The five-plate screening test for antibiotic residues is based on the use of five test plates prepared following the standard operating procedure of the National Centre for Food laboratory (AESAN, 2012) (Table 1).

Table 1. Test plates of the five plates screening technique: summary of their preparation and incubation

Plate	pH 6	DST	pH 8	EC	KR
Microorganism	*Bacillus subtilis* BGA Merck 1.10649	*Bacillus subtilis* BGA Merck 1.10649	*Bacillus subtilis* BGA Merck 1.10649	*Escherichia coli* CECT 4201	*Kocuria rhizophila* CECT 241
Culture medium	Testagar pH 6 Merck 1.10663	Diagnostic Sensitivity Test Oxoid CM 261	Testagar pH 8 Merck 1.10664	Testagar pH 8 Merck 1.10664	Testagar pH 8 Merck 1.10664
ufc/ml	10^4	10^4	10^4	10^5	10^4
Others µg/ml	NO	Trimethoprim 0.03 µg/ml	NO	NO	NO
ml of medium per 90 or 150 nm plate	90 mm-10 ml 150 mm-25ml	90 mm-10 ml 150 mm-25ml	90 mm-10 ml 150 mm-25ml	90 mm-10 ml 150 mm-25ml	90 mm-10 ml 150 mm-25ml
Control disc	Penicillin 0.01U/disc	Sulfadimidine 0.5 µg/disc	Streptomycin 0.5 µg/disc	Ciprofloxacin 0.003 µg/disc	Streptomycin 0.5 µg/disc
Result of control disc (inhibition zone)	> 6 mm wide	> 6 mm wide	> 6 mm wide	> 6 mm wide	>4 mm wide
Incubation temperature	30 ºC	30 ºC	30 ºC	30 ºC	37 ºC
Incubation time	18-24 hours	18-24 hours	18-24 hours	18-24 hours	18-24 hours

4. Assay procedure

When the plates are ready, the samples are prepared.

For samples of tissue, two tissue discs are applied on each of the test plates. These discs are obtained with a punch of 8 mm in internal diameter and have 2 mm in height. Fluid samples such as milk or egg are placed in 8 mm diameter wells perforated until reaching the bottom of the culture medium.

The plates are incubated following the assay procedure (see Table 1) and the width of the growth inhibition zone obtained is measured to determine whether the result is positive or not. The result is positive if the width of the inhibition zone is equal to or greater than 2 mm. A control disc is placed in the centre of the plates to verify whether the sensitivity of the plate is adequate in each test.

5. Assessment of specificity

Although the technique is necessarily unspecific because it only attempts to detect inhibitors of bacterial growth, some types of samples may contain natural inhibitors and lead to confirm the presence of antibiotic residues in samples which, in fact, do not contain them. This does not represent a problem from the legal point of view in Spain, because any positive result must be confirmed by more specific techniques. However, it is not desirable from an operative point of view for systematic interferences to occur due to natural inhibitors or other substances which are usually present in samples.

For example, pig kidney contains natural inhibitors whose activity can be prevented by placing a cellulose membrane between the tissue and the culture medium (Calderón et., 1992). Eggs also contain natural inhibitors and, in order to reduce their activity, samples are heat treated at 70 ºC for 20 minutes.

6. Estimation of detection limit

As there are maximum residue limits (MRL) legislated for antibiotic residues (Regulation (EC) No 470/2009) the suitability of the detection limits of the technique to these maximum limits must be studied and, to do so, we conduct tests with negative samples to which an antibiotic solution is added.

To add the antibiotic to fluid samples such as milk or eggs, the antibiotic solution is added straight to the sample and homogenized. However, in tissue samples, where homogenizations or extractions cannot be performed because the whole tissue is used, a problem arises over adding the antibiotic to make it representative of the presence of antibiotics in the tissue of a treated animal.

Regarding this, the Guide published by the European Union Reference Laboratory for antibiotic residues (ANSES, 2010) offers two possibilities:

- The tissue is homogenized, weighed, the antibiotic is then added and it is mixed. It is then frozen. The pieces of tissue with the antibiotic added and frozen are applied directly onto the plate.
- Paper discs are impregnated with the antibiotic for testing, they are placed on the plate on the culture medium and, then, the discs of whole tissue are placed on top of these paper discs, without homogenizing.

In this paper we have studied the two possibilities proposed by the European Union's Reference Laboratory. To do so, the steps followed for the preparation of samples added in each case are:

- Homogenize the tissue without antibiotic, add a dilution of antibiotic (0.1 ml in 5 g of sample) and

mix it. Once mixed, it is introduced into a stainless steel cylinder, 8 mm internal diameter, and it is frozen at -20 ºC. After this it is analyzed in the same way as the intact tissue samples.

- Use a paper disc impregnated with the antibiotic solution on which a disc of intact tissue is placed. To do this, in our case, we applied 20 µl of an antibiotic solution on a 6 mm diameter paper disc for antibiograms; the disc is placed on the test plate and on top of it we apply a 2 mm high intact tissue disc free of antibiotics obtained with an 8 mm internal diameter punch. The tissue disc has an approximate mean weight of 0.1 g and, for this reason, the quantity of antibiotic present is extrapolated to the weight of the disc of tissue (0.1 g) to establish the concentration added to the matrix (µg/kg).

Results

This paper has estimated the detection limits of the five-plate screening technique. First of all, we used pattern solutions to determine in which plate each antibiotic was more active and, therefore, produced a greater inhibition zone. We then determined the detection limit for a group of 23 antibiotics with each of the two estimation systems (tissue homogenized with a solution of antibiotic or intact tissue placed on a paper disc with antibiotic). Finally, we performed 20 trials to determine the detection limit of 57 antibacterial agents with samples of muscle from different species, bovine kidney, milk, eggs and fish. We performed the trials combining different conditions including factors such as day of trials, analysts, samples or batches of culture media preparation and reagents.

1. Determination of the plate most sensitive to each antibiotic

We had a previous study about the sensitivity of each of the five plates which featured the technique for different solutions of antibiotics (Calderón, 2000).

Taking into account the results of that study, the validation of each antibiotic only took place on the plate where the inhibition zone was greater than a specific concentration of each antibiotic.

In general, and bearing in mind that there were exceptions, the tetracyclines were detected on the plate with *B. subtilis* at pH 6, the beta-lactam antibiotics on the plate with *K. rhizophila* at pH 8 or on that of *B. subtilis* at pH 6, the cephalosporins on the plate with *K. rhizophila* at pH 8, the aminoglycosides on that of *B. subtilis* at pH 8, the macrolides on the plate with *K. rhizophila* and the quinolones on the plate with *E. coli* at pH 8.

2. Comparison of the estimation systems of the detection limit

Tables 2 and 3 compare the detection limits obtained with both estimation systems for a group of 23 antibiotics in porcine muscle and bovine kidney. We observed that, in general, the estimated detection limit was greater when tissue homogenized with a solution of antibiotic was used compared to the system combining a disc of intact tissue and a paper disc with antibiotic.

Okerman et al. (2004) observed that when homogenizing samples of chicken muscle which contained tetracyclines (doxycycline) the inhibition zones were smaller than those obtained with the same samples which had not been homogenized. This reduction may be due to the union of the antibiotic to the homogenized tissue which leads to an underestimation of the detection limits, at least for certain

groups of antibiotics. The results of the confirmation by physicochemical methods of positive samples on tetracyclines in the National Centre for Food indicated that the real detection limits of the five-plate screening technique for this group of antibiotics was closer to those obtained with the combination of antibiotic discs and whole tissue discs than those obtained with tissue homogenized with antibiotic.

As a result, the system combining antibiotic discs and whole tissue discs was chosen to perform the estimation of the detection limit in the validation process for this technique.

Table 2. Comparison of the detection limits (DL) obtained with porcine muscle with the two systems of estimation

Antibiotic	MRL (µg/kg)	DL paper disc + tissue (µg/kg)	DL homogenized tissue (µg/kg)
Chlortetracycline	100	80	200
Amoxicilin	50	50	100
Ampicillin	50	50	80
Benzylpenicillin	50	24	80
Oxacilin	300	300	400
Danofloxacino	100	80	200
Difloxacino	400	240	300
Enrofloxacino	100	30	50
Marbofloxacino	150	30	60

Table 3. Comparison of the detection limits (DL) obtained with bovine kidney with the two systems of estimation

Antibiotic	MLR (µg/kg)	DL paper disc + tissue (µg/kg)	DL homogenized tissue (µg/kg)
Chlortetracycline	600	80	280
Doxycycline	600	160	320
Oxytetracycline	600	600	800
Tetracycline	600	400	600
Benzylpenicillin	50	24	80
Nafcillin	300	100	120
Oxacillin	300	300	400
Cephalexin	1,000	1,000	1,600
Cefquinome	200	160	180
Apramycin	20,000	10,000	20,000
Gentamicin	750	600	1,600
Kanamicin	2,500	2,000	8,000
Neomycin	5,000	4,000	10,000
Erythromycin	200	140	200
Lincomycin	1,500	800	800
Tilmicosin	1,000	240	480
Difloxacin	800	120	200
Enrofloxacin	200	10	16
Flumequine	1,500	600	1,400
Marbofloxacin	150	14	12

3. Determination of the detection limit

The detection limit was established in the concentration which caused inhibition zones equal to or greater than 2 mm in width. However, the inhibition zones of some antibiotics do on occasions not have a clearly defined edge so that the width of the inhibition area used to establish the detection limit may have exceeded 2 mm in width depending on the definition of the edge of the zone in each case. Thus, the mean width of zone used to establish the detection limit was 5.09 mm considering all the antibiotics and all the matrices and of 4.62 mm if the sulphonamides are excluded, because their inhibition zone edges are very diffuse.

To establish the detection limit we took into account the criteria established by Decision 2002/657/EC so that the percentage of false negative results must be below 5% at the detection limit level.

Tables 4 to 19 show the results of the estimation of the detection limit of the five-plate screening technique in samples of porcine, bovine, ovine and poultry muscle and bovine kidney, milk, egg and fish. The results come from 20 samples for each antibiotic in each of the matrices.

Table 4. Detection limit for tetracyclines in muscle

Tetracyclines	MRL µg/kg porcine	Porcine muscle DL µg/kg[3]	MRL µg/kg bovine	Bovine muscle DL µg/kg	MRL µg/kg ovine	Ovine muscle DL µg/kg	MRL µg/kg poultry	Poultry muscle DL µg/kg
Detection plate		**IZ[1] (mm)±SD[2]**		**IZ (mm)±SD**		**IZ (mm)±SD**		**IZ (mm)±SD**
Chlortetracycline pH 6	100	80 3.63±0.47	100	100 5.28±0.59	100	80 4.82±0.36	100	100 6.22±0.82
Doxycycline pH 6	100	140 3.97±0.57	100	100 3.79±1.10	No limit	- -	100	100 3.39±0.51
Oxytetracycline pH 6	100	500 3.81±0.46	100	400 3.60±0.47	100	400 4.59±0.92	100	400 3.69±0.56
Tetracycline pH 6	100	300 3.45±0.50	100	300 4.86±1.72	100	400 3.79±0.45	100	300 3.36±0.70

[1]IZ: Inhibition zones. mean width (mm).

[2]SD: Standard deviation.

[3]µg/kg (ppb).

Table 5. Detection limit for beta-lactams in muscle

Beta-lactams	MLR µg/kg porcine	Porcine muscle DL µg/kg	MLR µg/kg bovine	Bovine muscle DL µg/kg	MLR µg/kg ovine	Ovine muscle DL µg/kg	MLR µg/kg poultry	Poultry muscle DL µg/kg
Detection Plate		**IZ (mm)±SD**		**IZ (mm)±SD**		**IZ (mm)±SD**		**IZ (mm)±SD**
Amoxicillin	50	50	50	50	50	50	50	50
KR		2.71±0.27		3.87±1.32		3.60±0.55		3.41±0.58
Ampicillin	50	50	50	50	50	50	50	50
KR		2.90±0.22		4.07±0.55		4.76±0.40		3.89±0.51
Benzylpencillin	50	24	50	20	50	40	50	50
pH 6		3.79±0.45		4.49±0.72		5.76±0.82		6.14±0.98
Cloxacillin	300	800	300	600	300	600	300	1,400
pH 6		3.83±0.66		4.29±0.45		4.18±0.59		4.71±0.86
Dicloxacillin	300	500	300	300	300	300	300	600
pH 6		4.33±0.94		3.22±0.59		3.80±1.60		4.47±1.21
Phenoxymethyl-		30	No	-	No	-		25
penicillin	25						25	
pH 6		3.76±0.73	limit	-	limit	-		3.70±0.56
Nafcillin	No	-	300	100	300	100	No	-
KR	limit	-		5.15±0.66		5.47±0.38	limit	-
Oxacillin	300	300	300	300	300	300	300	300
KR		3.60±0.50		3.82±0.46		4.76±0.54		3.68±0.74

Table 6. Detection limit for cephalosporins in muscle

Cephalosporins	MLR µg/kg porcine	Porcine muscle DL µg/kg	MLR µg/kg bovine	Bovine muscle DL µg/kg	MLR µg/kg ovine	Ovine muscle DL µg/kg	MLR µg/kg poultry	Poultry muscle DL µg/kg
Detection Plate		**IZ (mm)±SD**		**IZ (mm)±SD**		**IZ (mm)±SD**		**IZ (mm)±SD**
Cephalexin	No	-	200	400	No	-	No	-
KR	limit	-		3.06±0.46	limit	-	limit	-
Cephapirin	No	-	50	1,000	No	-	No	-
KR	limit	-		4.76±0.59	limit	-	limit	-
Cefquinome	50	160	50	200	No	-	No	-
KR		4.90±0.78		3.28±0.48	limit	-	limit	-
Ceftiofur	1,000	2,000	1,000	1,000	1,000	1,000	No	-
KR		4.77±1.06		5.51±1.31		3.29±0.72	limit	-

Table 7. Detection limit for aminoglicosydes in muscle

Aminoglycosides	MLR µg/kg porcine	Porcine muscle DL µg/kg	MLR µg/kg bovine	Bovine muscle DL µg/kg	MLR µg/kg ovine	Ovine muscle DL µg/kg	MLR µg/kg poultry	Poultry muscle DL µg/kg
Detection Plate		**IZ (mm)±SD**		**IZ (mm)±SD**		**IZ (mm)±SD**		**IZ (mm)±SD**
Apramycin	No	-	1,000	10,000	No	-	No	-
pH 8	limit	-		3.41±0.49	limit	-	limit	-
Dihydrostreptomycin	500	10,000	500	20,000	500	30,000	No	-
pH 8		4.13±0.37		5.57±0.47		7.07±0.86	limit	-
Spectinomycin	300	200,000	300	140,000	300	140,000	300	140,000
KR		4.81±1.13		5.55±0.56		5.28±0.80		4.92±0.96
Streptomycin	500	10,000	500	10,000	500	10,000	No	-
pH 8		5.05±0.55		3.71±1.16		4.95±0.92	limit	-
Gentamicin	50	1,800	50	2,000	No	-	No	-
pH 8		4.28±1.18		4.22±0.42	limit	-	limit	-
Kanamycin	100	5,000	100	6,000	100	10,000	100	8,000
pH 8		4.43±0.44		4.30±0.52		4.74±0.36		4.61±0.55
Neomycin	500	10,000	500	10,000	500	10,000	500	10,000
pH 8		4.43±0.77		4.53±0.75		4.53±0.38		4.50±1.01
Paromomycin	500	8,000	500	10,000	500	10,000	500	6,000
pH 8		4.19±0.52		4.02±0.87		4.09±0.44		3.09±0.39

Table 8. Detection limit for macrolides in muscle

Macrolides	MLR µg/kg porcine	Porcine muscle DL µg/kg	MLR µg/kg bovine	Bovine muscle DL µg/kg	MLR µg/kg ovine	Ovine muscle DL µg/kg	MLR µg/kg poultry	Poultry muscle DL µg/kg
Detection Plate		**IZ (mm)±SD**		**IZ (mm)±SD**		**IZ (mm)±SD**		**IZ (mm)±SD**
Erytromycin	200	200	200	200	200	200	200	200
KR		4.68±0.21		2.92±1.31		3.32±1.16		3.51±1.03
Spiramicin	250	7,000	200	2,000	No	-	200	2,000
KR		7.05±1.85		5.81±1.23	limit	-		4.45±0.97
Lincomycin	100	2,000	100	2,000	100	2,000	100	2,000
KR		5.31±1.30		5.89±1.56		4.79±0.64		5.12±0.74
Tilmicosin	50	1,800	50	1,000	50	1,000	75	1,000
KR		5.81±1.29		6.10±2.81		6.23±0.97		8.80±1.54
Tylosin	100	1,400	100	800	100	800	100	800
KR		3.83±0.87		4.41±1.19		4.47±0.72		5.19±0.73

Table 9. Detection limit for quinolones in muscle

Quinolones	MLR µg/kg porcine	Porcine muscle DL µg/kg	MLR µg/kg bovine	Bovine muscle DL µg/kg	MLR µg/kg ovine	Ovine muscle DL µg/kg	MLR µg/kg poultry	Poultry muscle DL µg/kg
Detection Plate		**IZ (mm)±SD**		**IZ (mm)±SD**		**IZ (mm)±SD**		**IZ (mm)±SD**
Oxolinic acid EC	100	300 4.55±1.25	100	2,000 6.74±0.52	100	1,000 5.43±0.53	100	1,000 4.78±0.68
Danofloxacin EC	100	80 5.79±1.14	200	100 5.98±0.87	200	160 6.01±0.54	200	160 6.18±0.80
Difloxacin EC	400	240 3.95±0.33	400	400 3.15±0.35	400	400 3.40±0.36	300	400 3.14±0.34
Enrofloxacin EC	100	30 4.34±0.28	100	60 4.74±1.40	100	80 5.91±0.48	100	60 4.23±0.62
Flumequine pH 6	200	600 4.61±0.84	200	400 4.16±0.84	200	800 5.96±0.64	400	400 3.86±0.75
Marbofloxacin EC	150	30 3.68±0.7	150	150 6.22±0.65	No limit	- -	No limit	- -

Table 10. Detection limits for other antibiotics in muscle

Other antibiotics	MLR µg/kg porcine	Porcine muscle DL µg/kg	MLR µg/kg bovine	Bovine muscle DL µg/kg	MLR µg/kg ovine	Ovine muscle DL µg/kg	MLR µg/kg poultry	Poultry muscle DL µg/kg
Detection Plate		**IZ (mm)±SD**		**IZ (mm)±SD**		**IZ (mm)±SD**		**IZ (mm)±SD**
Colistin EC	150	160,000 4.30±0.48	150	100,000 4.26±0.40	150	100,000 4.56±0.30	150	100,000 4.51±0.33
Florfenicol pH 6	300	7,000 4.97±0.59	200	8,000 5.71±0.46	200	8,000 5.09±0.49	100	8,000 5.14±0.38
Florfenicol pH 8	300	7,000 4.62±0.53	200	8,000 5.10±0.63	200	8,000 4.86±0.53	100	8,000 5.68±0.41
Monensin pH 6	No limit	- -	2	14,000 3.15±0.31	No limit	- -	No limit	- -
Tiamulin KR	100	2,800 5.54±1.27	No limit	- -	No limit	- -	100	1,600 5.14±0.96
Thiamphenicol KR	50	30,000 3.96±0.75	50	20,000 3.75±0.73	50	20,000 3.64±0.76	50	20,000 3.70±0.74
Trimethoprim DST	100	800 7.88±0.80	50	2,000 10.45±1.44	50	2,000 9.60±0.54	50	2,000 11.21±1.35
Valnemulin KR	50	800 7.00±0.42	No limit	- -	No limit	- -	50	800 4.96±1.10

Table 11. Detection limits for sulfonamides in muscle

Sulfonamides	MRL µg/kg porcine	Porcine muscle DL µg/kg	Bovine muscle DL µg/kg	Ovine muscle DL µg/kg	Poultry muscle DL µg/kg
Detection plate		**IZ (mm) ± SD**	**IZ (mm) ± SD**	**IZ (mm) ± SD**	**IZ (mm) ± SD**
Sulfachlorpyridazine	100	8,000	4,000	3,000	4,000
DST		8.80±1.36	8.49±0.99	6.43±1.07	8.26±1.41
Sulfadiazine	100	4,000	2,000	2,000	2,000
DST		10.02±1.47	9.28±1.33	9.98±0.66	9.41±0.90
Sulfadimethoxine	100	2,000	2,000	2,000	1,000
DST		6.62±0.95	10.95±0.81	10.45±1.19	8.17±0.96
Sulfadimidine	100	10,000	4,000	4,000	4,000
DST		9.23±1.73	8.14±3.00	5.53±0.56	8.47±2.27
Sulfamethoxazol	100	2,000	1,000	1,000	1,000
DST		10.03±1.28	8.92±2.30	5.41±0.92	8.54±1.82
Sulfamethoxypyridazine	100	6,000	2,000	2,000	2,000
DST		10.18±1.17	8.91±1.31	7.29±0.80	6.73±0.61
Sulfapyridine	100	8,000	4,000	4,000	4,000
DST		10.49±1.58	9.74±1.04	6.29±0.56	10.04±0.36
Sulphaquinoxaline	100	4,000	1,000	1,000	1,000
DST		11.05±1.28	9.31±0.64	7.98±0.71	10.23±1.03
Sulfathiazole	100	3,000	1,000	2,000	3,000
DST		8.89±2.24	5.00±0.41	8.76±1.44	9.53±1.88

Table 12. Detection limits for tetracyclines in bovine kidney, milk, egg and fish

Tetracyclines	MRL µg/kg kidney	Bovine kidney DL µg/kg	MRL µg/kg milk	Milk DL µg/kg	MRL µg/kg egg	Egg DL µg/kg	MRL µg/kg fish	Fish DL µg/kg
Detection plate		**IZ (mm)±SD**		**IZ (mm)±SD**		**IZ (mm)±SD**		**IZ (mm)±SD**
Chlortetracycline	600	80	100	100	200	1,500	100	100
pH 6		2.86±0.52		3.44±1.03		5.14±0.56		4.61±0.99
Doxycycline	600	160	No	-	No	-	No	-
pH 6		3.70±0.49	limit	-	limit	-	limit	-
Oxytetracycline	600	600	100	600	200	3,000	100	600
pH 6		3.46±0.43		3.56±0.48		4.31±0.66		4.56±1.15
Tetracycline	600	400	100	800	200	3,000	100	400
pH 6		3.55±0.58		4.67±0.96		4.22±0.38		3.70±0.44

Table 13. Detection limits for beta-lactams in bovine kidney, milk, egg and fish

Beta-lactams	MRL µg/kg kidney	Bovine kidney DL µg/kg	MRL µg/kg milk	Milk DL µg/kg	MRL µg/kg egg	Egg DL µg/kg	MRL µg/kg fish	Fish DL µg/kg
Detection plate		**IZ (mm)±SD**		**IZ (mm)±SD**		**IZ (mm)±SD**		**IZ (mm)±SD**
Amoxicillin	50	80	4	100	No	-	50	100
KR		3.87±0.46		6.11±0.51	limit	-		5.30±0.68
Ampicillin	50	80	4	100	No	-	50	40
KR		4.99±1.18		7.86±1.05	limit	-		3.50±0.48
Benzylpenicillin	50	24	4	50	No	-	50	24
pH 6		3.44±0.61		5.69±0.37	limit	-		3.69±0.78
Cloxacillin	300	1,200	30	1,000	No	-	300	1,600
pH 6		4.15±1.00		3.92±0.83	limit	-		5.03±1.11
Dicloxacillin	300	600	30	500	No	-	300	500
pH 6		4.83±0.54		4.62±1.65	limit	-		3.78±0.70
Nafcillin	300	100	30	100	No	-	No	-
KR		3.90±0.51		4.62±0.65	limit	-	limit	-
Oxacillin	300	300	30	300	No	-	300	300
KR		4.16±0.54		4.82±0.55	limit	-		4.81±0.52

Table 14. Detection limits for cephalosporins in bovine kidney, milk, egg and fish

Cephalosporins	MRL µg/kg kidney	Bovine kidney DL µg/kg	MRL µg/kg milk	Milk DL µg/kg	MRL µg/kg egg	Egg DL µg/kg	MRL µg/kg fish	Fish DL µg/kg
Detection plate		**IZ (mm)±SD**		**IZ (mm)±SD**		**IZ (mm)±SD**		**IZ (mm)±SD**
Cephalexin	1,000	1,000	100	1,000	No	-	No	-
KR		4.60±0.99		6.26±1.72	limit	-	limit	-
Cefalonium	No	-	20	500	No	-	No	-
KR	limit	-		8.37±0.61	limit	-	limit	-
Cephapirin	100	-	60	60	No	-	No	-
KR		-		4.31±0.52	limit	-	limit	-
Cefazolin	No	-	50	4,000	No	-	No	-
KR	limit	-		5.45±1.17	limit	-	limit	-
Cefoperazone	No	-	50	1,000	No	-	No	-
KR	limit	-		5.33±0.99	limit	-	limit	-
Cefquinome	200	160	20	200	No	-	No	-
KR		5.20±0.53		3.36±0.91	limit	-	limit	-
Ceftiofur	6,000	6,000	100	200	No	-	No	-
KR		6.23±1.43		5.50±1.73	limit	-	limit	-

Table 15. Detection limits for aminoglycosides in bovine kidney, milk, egg and fish

Aminoglycosides	MRL µg/kg kidney	Bovine kidney DL µg/kg	MRL µg/kg milk	Milk DL µg/kg	MRL µg/kg egg	Egg DL µg/kg	MRL µg/kg fish	Fish DL µg/kg
Detection plate		**IZ (mm)±SD**		**IZ (mm)±SD**		**IZ (mm)±SD**		**IZ (mm)±SD**
Apramycin	20,000	10,000	No	-	No	-	No	-
pH 8		3.64±0.39	limit	-	limit	-	limit	-
Dihydrostreptomycin	1,000	3,000	200	3,000	No	-	No	-
pH 8		3.31±0.41		4.00±0.69	limit	-	limit	-
Spectinomycin	5,000	120,000	200	30,000	No	-	300	200,000
KR		4.22±0.92		4.30±0.73	limit	-		6.72±1.20
Streptomycin	1,000	3,000	200	5,000	No	-	No	-
pH 8		3.73±0.63		5.35±0.94	limit	-	limit	-
Gentamicin	750	600	100	200	No	-	No	-
pH 8		4.17±0.58		2.80±0.37	limit	-	limit	-
Kanamycin	2,500	2,000	150	2,000	No	-	No	-
pH 8		3.44±0.58		4.49±0.66	limit	-	limit	-
Neomycin	5,000	4,000	1,500	3,000	500	7,000	500	14,000
pH 8		4.33±0.76		4.21±0.37		3.91±0.57		4.29±0.52
Paromomycin	1,500	2,600	No	-	No	-	500	10,000
pH 8		4.05±0.56	limit	-	limit	-		3.94±0.62

Table 16. Detection limits for macrolides in bovine kidney, milk, egg and fish

Macrolides	MRL µg/kg kidney	Bovine kidney DL µg/kg	MRL µg/kg milk	Milk DL µg/kg	MRL µg/kg egg	Egg DL µg/kg	MRL µg/kg fish	Fish DL µg/kg
Detection plate		**IZ (mm)±SD**		**IZ (mm)±SD**		**IZ (mm)±SD**		**IZ (mm)±SD**
Erytromycin	200	140	40	40	150	100	200	320
KR		4.08±0.93		5.25±0.94		4.55±0.81		4.59±1.29
Spiramycin	300	1,400	200	200	No	-	No	-
KR		3.42±0.60		3.79±0.78	limit	-	limit	-
Lincomycin	1,500	800	150	800	50	500	100	1,400
KR		4.43±0.49		5.00±0.81		3.66±0.42		5.44±0.81
Tilmicosin	1,000	240	50	50	No	-	50	1,000
KR		3.74±1.04		5.38±1.01	limit	-		5.73±1.35
Tylosin	100	800	50	300	200	800	100	1,200
KR		3.94±0.29		4.25±1.18		3.52±0.33		4.52±0.44

Table 17. Detection limits for quinolones in bovine kidney, milk, egg and fish

Quinolones	MRL µg/kg kidney	Bovine kidney DL µg/kg	MRL µg/kg milk	Milk DL µg/kg	MRL µg/kg egg	Egg DL µg/kg	MRL µg/kg fish	Fish DL µg/kg
Detection plate		**IZ (mm)±SD**		**IZ (mm)±SD**		**IZ (mm)±SD**		**IZ (mm)±SD**
Oxolinic acid EC	150	300 4.39±1.08	No limit	- -	No limit	- -	100	280 3.64±0.75
Danofloxacin EC	400	200 8.51±0.93	30	30 4.01±0.61	No limit	- -	100	80 5.80±0.58
Difloxacin EC	800	120 3.97±0.48	No limit	- -	No limit	- -	300	90 5.02±0.67
Enrofloxacin EC	200	10 5.03±0.97	100	80 7.27±0.85	No limit	- -	100	6 4.44±0.42
Flumequine pH 6	1,500	600 3.82±0.66	50	600 4.97±0.59	No limit	- -	600	660 4.41±0.39
Marbofloxacin EC	150	14 5.19±1.02	75	100 6.75±0.73	No limit	- -	No limit	- -
Sarafloxacin EC	No limit	- -	No limit	- -	No limit	- -	30	200 4.69±0.60

Table 18. Detection limits for others antibiotics in bovine kidney, milk, egg and fish

Other antibiotics	MRL µg/kg kidney	Bovine kidney DL µg/kg	MRL µg/kg milk	Milk DL µg/kg	MRL µg/kg egg	Egg DL µg/kg	MRL µg/kg fish	Fish DL µg/kg
Detection plate		**IZ (mm)±SD**		**IZ (mm)±SD**		**IZ (mm)±SD**		**IZ (mm)±SD**
Bacitracin KR	No limit	- -	100	2,000 4.56±1.00	No limit	- -	No limit	- -
Colistin EC	200	60,000 5.14±0.89	50	10,000 2.94±0.61	300	50,000 3.70±0.83	150	60,000 4.27±0.56
Florfenicol pH 6	300	6,000 4.10±1.08	No limit	- -	No limit	- -	1,000	7,000 4.94±0.62
Florfenicol pH 8	300	6,000 3.92±0.97	No limit	- -	No limit	- -	1,000	7,000 4.41±0.49
Novobiocin KR	No limit	- -	50	10,000 4.85±0.57	No limit	- -	No limit	- -
Tiamulin KR	No limit	- -	No limit	- -	1,000	3,500 4.53±0.51	No limit	- -
Tiamphenicol KR	50	30,000 4.45±0.63	50	40,000 7.03±1.52	No limit	- -	50	30,000 3.94±0.41
Trimethoprim DST	50	600 9.08±1.01	50	500 6.43±0.63	No limit	- -	50	400 4.97±1.29

Table 19. Detection limits for sulfonamides in bovine kidney, milk, egg and fish

Sulfonamides	MRL µg/kg kidney	Bovine kidney DL µg/kg	MRL µg/kg milk	Milk DL µg/kg	MRL µg/kg egg	Egg DL µg/kg	MRL µg/kg fish	Fish DL µg/kg
Detection plate		**IZ (mm)±SD**		**IZ (mm)±SD**		**IZ (mm)±SD**		**IZ (mm)±SD**
Sulfachlorpyridazine	100	10,000	100	5,000	No	-	100	4,000
DST		10.17±0.80		9.75±0.98	limit	-		7.85±1.77
Sulfadiazine	100	4,000	100	500	No	-	100	2,000
DST		10.26±1.03		5.78±2.02	limit	-		7.92±1.53
Sulfadimethoxine	100	4,000	100	500	No	-	100	800
DST		9.88±1.33		6.34±1.68	limit	-		5.74±2.41
Sulfadimidine	100	10,000	100	5,000	No	-	100	4,000
DST		10.07±1.27		9.38±2.17	limit	-		6.35±1.16
Sulfamethoxazol	100	2,000	100	500	No	-	100	800
DST		8.92±1.13		6.35±1.38	limit	-		6.93±1.54
Sulfamethoxypyridazine	100	4,000	100	2,000	No	-	100	2,000
DST		8.99±1.43		9.51±0.68	limit	-		7.20±1.41
Sulfapyridine	100	8,000	100	2,000	No	-	100	4,000
DST		10.18±1.26		8.32±0.80	limit	-		7.39±1.75
Sulphaquinoxaline	100	4,000	100	500	No	-	100	800
DST		10.52±0.79		4.87±1.22	limit	-		6.24±1.89
Sulfatiazole	100	2,000	100	800	No	-	100	1,000
DST		7.97±1.75		6.13±1.45	limit	-		5.67±1.13

Tabla 20. Comparison of the detection limit (DL) compared to the maximum residue limit (MRL) of the tested antibiotics (number of antibiotics)

	LD ≤ LMR	1 LMR< LD ≤ 2 LMR	LD > 2 LMR	Total antibiotics tested
Porcine muscle	10	4	33	47
Bovine muscle	14	3	32	49
Ovine muscle	12	1	27	40
Poultry muscle	11	2	28	41
Bovine kidney	22	5	20	47
Milk	7	4	35	46
Egg	1	0	8	9
Fish	7	4	25	36

Table 21. Antibiotics whose detection limit was less than or equal to the maximum residue limit

Porcine muscle	Bovine muscle	Ovine muscle	Poultry muscle	Bovine kidney	Milk	Egg	Fish
Chlortetracycline	Chlortetracycline	Chlortetracycline	Chlortetracycline	Chlortetracycline	Chlortetracycline	Eritromicyn	Chlortetracycline
Amoxicillin	Doxicyclin	Amoxicillin	Doxicyclin	Doxicyclin	Cephapyrin		Ampicillin
Ampicillin	Amoxicillin	Ampicillin	Amoxicillin	Oxytetracyclin	Erytromycin		Benzylpenicillin
Benzylpenicillin	Ampicillin	Benzylpenicillin	Ampicillin	Tetracyclin	Espiramycin		Oxacillin
Oxacillin	Benzylpenicillin	Dicloxacillin	Benzylpenicillin	Benzylpenicillin	Tilmicosin		Danofloxacin
Erytromycin	Dicloxacillin	Nafcillin	Phenoxymethyl-	Nafcillin	Danofloxacin		Difloxacin
Danofloxacin	Nafcillin	Oxacillin	penicillin	Oxacillin	Enrofloxacin		Enrofloxacin
Difloxacin	Oxacillin	Ceftiofur	Oxacillin	Cephalexin			
Enrofloxacin	Ceftiofur	Erytromycin	Erytromycin	Cefquinome			
Marbofloxacin	Erytromycin	Danofloxacin	Danofloxacin	Ceftiofur			
	Danofloxacin	Difloxacin	Enrofloxacin	Apramycin			
	Difloxacin	Enrofloxacin	Flumequine	Gentamicin			
	Enrofloxacin			Kanamicin			
	Marbofloxacin			Neomicin			
				Eritromycina			
				Lincomycin			
				Tilmicosin			
				Danofloxacin			
				Difloxacin			
				Enrofloxacin			
				Flumequine			
				Marbofloxacin			

The bovine kidney was the matrix in which most antibiotics were detected at the level or under the maximum limit and the only one in which aminoglycoside antibiotics were detected. Chlortetracycline and danofloxacin were detected under their maximum residue limits in all the matrices tested except eggs. In turn, erythromycin was detected at the maximum residue limit in all matrices barring fish.

Within the tetracyclines, chlortetracycline and doxycycline were detected at lower levels than those of tetracycline and oxytetracycline. Among the beta-lactams, amoxicillin, ampicillin and penicillin G had lower detection limits than the rest.

The detection of beta-lactams by using this technique is not acceptable in milk given the frequency of these residues in this matrix. Other methods of detection should be used for their control such as the inhibition of the growth of *Bacillus stearothermophilus*, which are more sensitive to this group of antibiotics. The five-plate screening technique would only be useful in this matrix to detect those antibiotics which, like quinolones, are not well detected by *B. stearothermophilus*.

Quinolones form the group which is detected best with this technique in all matrices which present MRLs. This detection even occurs at levels which are far below the MRL so that subsequent identification and quantification by more specific confirmation techniques is important.

The detection of aminoglycosides and macrolides was clearly better in kidney than in muscle and no sulphonamides at all were detected at acceptable levels. This last group is analyzed using other techniques in the National Residue Plan so that its detection using this technique could only be expected in cases of very high contamination or matrices which, such as in animal feeds, may contain the active substance for therapeutic purposes.

Conclusions

The results obtained represent solely an estimation regarding the detection limits of the five-plate screening technique for antibiotic residues in tissues because only the use of samples of whole tissue would allow a precise estimation of its detection limits. Furthermore, the estimation has been performed with a high safety margin because we have only considered perfectly visible inhibition zones, easily surpassing the 2 mm wide threshold for the inhibition zone established in the technique to conclude a positive result.

The validation performed of the screening technique for antibiotic residues in conformity with the guidelines of Decision 2002/657/EC has shown that, although for some residues of antibiotics, detection is made at higher levels, this technique allows detection of a certain number of antibiotics in different foods at or below the level of their maximum residue limit. For these antibiotics, after analyzing 20 samples of porcine, bovine, ovine and poultry muscle, bovine kidney, milk, egg and fish, in all cases the samples were positive to the level of the maximum residue limit, causing an inhibition zone over 2 mm wide. It therefore complies with the criteria established in Decision 2002/657/EC, because the percentage of false negatives did not exceed 5% at this concentration, with the conclusion that this technique is valid for the intended use.

For those residues of antibiotics whose detection limits are insufficient and whose risk assesment establishes that they are of interest from a monitoring point of view, an alternative analytical strategy should be studied.

This technique is applicable for the detection of a wide range of antibiotic residues in different matrices of animal origin with a good specificity of the level of interest, a high applicability, as it is a simple and low-cost technique, with a detection limit equal or below the maximum residue limit for different antibiotic residues.

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