

Collaboration

Thirty years of applying microbiological methods for the control of antibiotic residues in the National Residue Monitoring Plan in foods in Spain

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Abstract

Microbiological methods for the analytical control of antibiotic residues are based on detecting the only common characteristic among all antibiotics, their ability to inhibit bacterial growth. Therefore, over the past 30 years in Spain, they have played an important role in the control of these residues within the framework of the National Residue Monitoring Plan in food.

Its simplicity, flexibility, low cost and multi-residue detection and sample processing capabilities made them the methods of choice for undertaking the analysis of antibiotic residue screening.

The validation of some of the antibiotic residue screening methods is complex given that intact tissue is used as a test sample. Therefore, the five-plate screening method validation was completed through collaborative trials in which the National Reference Laboratory and the official control laboratories participated, so that their validation was shared and the accreditation of the method was facilitated according to the ISO/IEC 17025 standard.

As a National Reference Laboratory for antibiotic residues in food, the National Centre for Food (CNA) of the Spanish Agency for Food Safety and Nutrition (AESAN) has carried out a whole series of activities to serve the official control laboratories, including: laboratory communications, conferences, courses, intercomparison and collaborative tests and supplying materials for analysis.

The current development of multi-residue physical-chemical instrumental methods and with detection limits better adjusted to the maximum antibiotic residue limits established in the European Union has resulted in a turning point and has limited the applicability of traditional microbiological methods for detecting antibiotic residues in the official control of the same.

Keywords

Antibiotics, residues, foods, inhibitors, microbiological methods, screening.

1. Introduction

2019 marks 30 years since the publication of Royal Decree 1262/1989, of 20 October, by which the National Residue Monitoring Plan in Animals and Fresh Meat, known as PNIR (BOE, 1989), was approved. This Royal Decree transposed Directive 86/469/EEC into national legislation, which sought to have sampling for the analysis of residues of pharmacological action substances and other products of zoosanitary use in animals and fresh meat be carried out according to common criteria in all of the then European Economic Community Member States (EU, 1986).

In Annex I of the Royal Decree the different groups of residues to be controlled were listed, including inhibitory substances in group A.III.a. Antibiotics, sulphonamides and similar antimicrobial substances.

In Annex II the levels and frequency of sampling were established, which, in the case of inhibitors should be done in 0.10 % of slaughtered animals, establishing that control of a pool of substances could be undertaken.

Furthermore, the National Reference Laboratories and authorised laboratories for conducting confirmation assessments were designated, and among them was the National Centre for Food and Nutrition of the Ministry of Health and Consumer Affairs. However, Royal Decree 1262/1989 only established National Reference Laboratories for hormonal substances and those having thyreostatic action, and for pesticides and heavy metals, but not for antibiotic residues. Commission Decision 89/610/EEC (EU, 1989) established the reference laboratories of the Member States but, in the case of Spain, it failed to identify for which residues the reference laboratory exercised. It was ultimately Commission Decision 93/257/EEC (EU, 1993a) that formally designated the National Centre for Food and Nutrition as the National Reference Laboratory for the residues from the aforementioned group A.III.a. Subsequently, Commission Decision 98/536/EC (EU, 1998) upheld this reference for the new group B1 (antibacterial substances, including sulphonamides, quinolones) created by Directive 96/23/EC.

There were also national programmes for the control of residues in food in non-EU countries. In the United States the National Residue Program was, and continues to be, managed by the Food Safety and Inspection Service (FSIS) of the Department of Agriculture (USDA) (Calderón, 1993).

Although the PNIR was the beginning of a systematic and planned official control at the national level of residues in meat, the existing regulation in the United States imposed a certification system for products, establishments and countries in order to allow the export of meat to its market. This involved establishing a control system for residues in meat in Spain prior to the PNIR, and in which the National Centre for Food (CNA) acted as a control laboratory and underwent FSIS audits.

This activity led to the development of microbiological methods for the control of antibiotic residues used in the United States at the time, and which were published in the FSIS Microbiology Laboratory Guidebook (USDA, 1974). These were microbiological screening methods, methods for identifying groups of antibiotics and even for quantifying these residues.

In 1980 an antibiotic residues screening method known as the four-plate test (Bogaerts and Wolf, 1980) was published in Europe, and it was used in numerous countries with different versions.

The regulation of residues in Europe was renewed based on the publication of Directive 96/23/EC (EU, 1996), which was transposed into Spanish legislation through Royal Decree 1749/1998

(BOE, 1998). Antibiotic residues were classified within group B1: antibacterial substances, including sulphonamides and quinolones, except those which were included in group A6 and whose use is prohibited, for example chloramphenicol.

The new PNIR created the National Coordinating Commission for Research and Control of Residues or Substances in Live Animals and their Products, replacing the former Inter-ministerial Commission for Researching Residues in Animals and Fresh Meat, and appointed the CNA as a National Reference Laboratory for antibiotic residues in accordance with the Laboratory for Veterinary Medicines of Fougères (France) (CNEVA-LMV, in French), currently within the French Agency for Food, Environmental and Occupational Health & Safety (ANSES), which was designated as a Community Reference Laboratory for antibiotic residues in 1991.

A significant new development of Royal Decree 1749/1998 is that it expanded the control of residues to other non-meat food products of animal origin, such as milk, eggs, honey and aquaculture products.

In the CNA, the activity as a National Reference Laboratory in this field was assigned, at that time, to the Bromatology Service, which had a Zoosanitary Residues Unit, and to the Microbiology Service, where microbiological methods for the control of antibiotic residues were developed in its Parasitology and Special Techniques Section.

2. Importance of controlling antibiotic residues

Antibiotics have been widely used in animals intended for food production (Díez and Calderón, 1997) and therefore they were one of the main residue groups to be controlled in the PNIR. Thus, in 1997 33 % of antibiotic consumption in the European Union was intended for therapeutic use in animals and 15 % for use as additives in feed, compared to 52 % used in human medicine (Fedesa, 1999). Antibiotics are often used in a legal manner, requiring the withdrawal periods to be respected, prior to slaughter or obtaining the foods necessary, in order to ensure that foods from the treated animal do not have any remaining residues that may harm consumers. Practically all foods of animal origin may contain antibiotic residues: meat, viscera and derivatives, milk and dairy products, fish and aquaculture products, eggs or honey. Furthermore, it is possible to control their presence in feed and zoosanitary products.

At that time, over 50 different antibiotics were used in veterinary medicine for therapeutic purposes (Veterindustria, 1997). There was also a large number of pharmaceutical specialties, 40 % of pharmaceutical products collected in a database developed by the then General Directorate of Livestock of the Ministry of Agriculture, Fisheries and Food contained antibiotics (Medicavet, 1997). Spain was a significant market for zoosanitary products. It held the fourth spot in Europe behind France, Germany and the United Kingdom, and held the seventh spot worldwide with 2.8 % of the global market (Veterindustria, 1999). It was the second largest consumer in the European Union of antibiotics for therapeutic use, which are the most important in generating residues (Boatman, 1998). Currently, according to the most recent data published by the European Medicines Agency (EMA), corresponding 30 European countries in 2016, Spain is the second largest consumer in terms of mg of active ingredient per PCU (population correction unit that takes into account corrections such as the weight of different categories of slaughtered animals or the number of animals exported or imported) (EMA, 2018).

The presence of antibiotic residues in food has significant repercussions for the food industry given that it could interfere in the production of foods such as yoghurt, cheese or cold meats, in which bacterial cultures partake. In turn, due to their toxicity and allergenic power, consumers may be affected by the presence of antibiotic residues in food.

Not only consumers, but the entire human and animal populations could suffer adverse consequences as a result of the indiscriminate use of antibiotics given that the antibiotic resistance selected by improper use may be transferred from some bacteria to others; from animal microbiota to that of humans, and vice versa.

Due to these problems, and the need to ensure the free exchange of foods through equivalent residue control systems in all European Union countries, programmes for the control of veterinary drug residues in foods were established.

Within the National Residue Plans, antibiotic residues were a very important group. Therefore, in 1995 the European Union collected 623 435 samples for the control of antibiotic residues compared to 91 545 for beta-agonists or 75 652 for hormonal compounds (PNIR, 1997). In Spain, the National Residue Monitoring Plan included the analysis of more than 65 000 samples for the control of different residues in 1999. Of these, 37 %, close to 24 000, were for the analysis of antibacterial compound residues (PNIR, 1999).

Currently, according to data from 2017 at the national level, more than 14 000 samples were analysed, representing 34.6 % of the samples of edible food matrices analysed that year.

At the EU level, the targeted or planned sampling of residues from antibacterial substances in 2017 involved the analysis of 109 206 samples, 30 % of the total, with 0.26 % of the results breaching the maximum residue limits (EFSA, 2019).

3. Microbiological methods for detecting antibiotic residues

The wide variety of antibiotic residues and the high number of samples to be controlled makes the use of screening techniques to detect them particularly interesting. In any event, there are currently 60 anti-infective drugs (antibiotics or chemotherapeutic) for which a maximum residue limit has been set. In total there are 344 maximum limits for these types of residues in different species and matrices, the majority in liver (76), kidney (72), and muscle (71), but also in fat (50), milk (46), skin and fat (17), eggs (9) and muscle and skin of fish (3) (EU, 2009).

Therefore, it is necessary to use a screening method that selects those samples that, initially, may have some antibiotic residue. A screening method must be economical and simple, given that it's applied to many samples; fast, because sometimes the carcass is detained or other post-screening, confirmatory or contradictory analyses or confirmation assessments must continue; multi-residue, able to detect a large number of compounds, since what is sought is a group of bacterial growth inhibitors and not a specific antibiotic; and with a detection limit that is properly adjusted to the maximum residue limit. It should be able to detect the residue at the maximum limit level in order to avoid false negative results, but not be overly sensitive in order to prevent false positive results, which lead to confirming samples that, from a legal standpoint, should not in fact pass the screening phase.

In this respect, microbiological methods have played an important role given that they are based on antibacterial activity, the only common trait among all antibiotics. It typically involves methods based on a culture of a sensitive bacterium that, if microorganism growth is inhibited when it encounters a food sample, there is a possibility that the sample contains an antibiotic residue.

The main method used in the CNA for the control of antibiotic residues in meat was the STOP (Swab Test On Premises) screening method from the United States (Johnston et al., 1981). Furthermore, commercial tests such as the Kundrat test (Kundrat, 1968, 1972) and the Brilliant Black Reduction (BR) test (Lloyd and Van der Merwe, 1987) were used. However, being the most commonly used in Europe, the four-plate test was soon used instead (Bogaerts and Wolf, 1980). This method, by using various microorganisms (*Bacillus subtilis* and *Micrococcus luteus*, now *Kocuria rhizophila*) in culture media of different pH for each of the test plates, creates favourable conditions for the antibacterial activity of different groups of antibiotics and, therefore, their detection. In addition, the treatment of the samples is not required given that they are placed directly, in the shape of tissue discs, on the surface of the inoculated culture media in Petri dishes.

Although the four-plate screening method was one of the most commonly used to detect antibiotic residues in meat, over time and in different countries other microbiological methods have been used, such as the Explorer test in Spain, which uses *Geobacillus stearothermophilus*, a pH indicator and an automatic colour reader.

In the case of milk, there is a long tradition in the industry of using tests based on the inhibition of *Geobacillus stearothermophilus*, a microorganism that is very sensitive to beta-lactam antibiotics and for which commercial tests were developed that offered results after 3 hours of incubation.

However, a positive result to the microbiological screening method does not make it possible to establish the identity and concentration of the residue, which also allows for the possibility that the positive result was due to some natural inhibitor present in the sample. Furthermore, given that the confirmation and quantification chromatographic techniques were different depending on the group of antibiotics, it was very useful to have information on the group of antibiotics which the residue could belong to in order to apply the appropriate confirmation method.

This linking function between screening and chromatographic confirmation was performed using the post-screening method by a multi-plate assay, in which the combination of microorganisms that are sensitive and resistant to different antibiotic groups with different culture media pH and a extraction with different buffer solutions allowed for the preliminary identification of the group of antibiotics the residue could belong to. This method, initially developed in the United States by the FSIS (USDA, 1974), was also originally used to quantify residues, albeit imprecisely (Calderón et al., 1994a).

The protocols for these methods were adapted and modified in the CNA in order to standardise procedures, improve the detection capacity of the methods and adjust them for their application to other food matrices.

As for the standardisation of procedures, different factors were studied that could influence the detection of residues such as homogenisation systems (Calderón et al., 1994b) or the measurement of inhibition zones (Calderón et al., 1995).

Among the screening method improvements is the introduction of a fifth plate with *E. coli* to enable the detection of quinolone residues (Ellerbroek, 1991), thus being called the five-plate method; the use of a cellulose membrane instead of a dialysis membrane to prevent the activity of natural pig kidney inhibitors (Figure 1) (Calderón et al., 1992a); or the treatment of egg and feed samples to make their analysis possible (CNA, 1998). One of the plates from Bogaerts and Wolf's (1980) original method was intended for detecting sulphonamide residues but its limit of detection was well above that required in order to detect residues at the established maximum limits, therefore it was ultimately rejected.

In the case of the post-screening method, the original FSIS protocol was simplified by using a monolayer of culture medium on the test plates instead of two layers, and the concentration of microorganisms on each plate was standardised. Plates were added in which the activity of certain groups of antibiotics, such as tetracyclines, was inhibited by using a resistant strain of *Bacillus cereus* (Aureli and Pasolini, 1984) or aminoglycoside by introducing heparin into the culture medium (Lund, 1986), in order to more easily identify these groups of antibiotics.

Furthermore, a validation of the post-screening microbiological method was done in order to determine the activity profiles of the antibiotics on each test plate and facilitate the identification of the antibiotic residues (Calderón et al., 1996a).

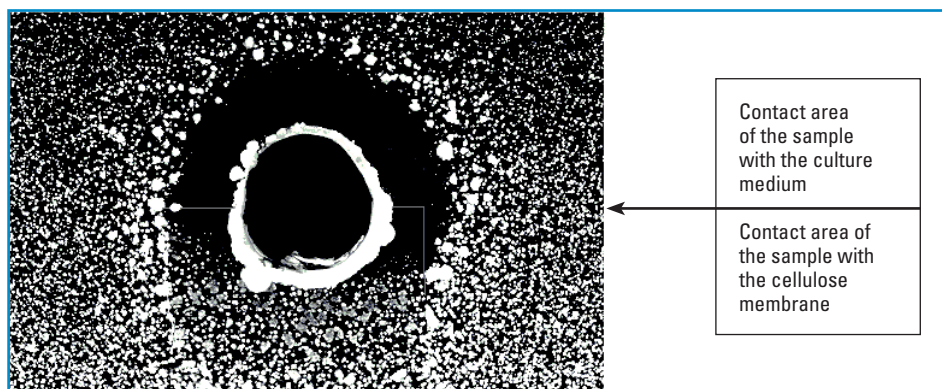


Figure 1. Reduction in the natural inhibition of a pig kidney by placing a cellulose membrane between the sample and the culture medium. Photo: G. Jiménez.

The protocols for the methods were provided to the regional monitoring laboratories and disclosed in different publications (Calderón, 1989) (Berenguer and Calderón, 1991) (Calderón, 1991a, b, 1992b, 2000a) (Díez and Calderón, 1991) (Díez et al., 2012a).

In other European Union countries different microbiological screening methods for antibiotic residues were used, based on the four-plate test or alternative systems such as the detection of antibiotics in renal pelvic fluid in the NDKT (New Dutch Kidney Test) and NAT (Nouws Antibiotic Test) (Nouws, 1988) methods developed in the Netherlands (Pikkemaat et al., 2008). The Community Reference Laboratory developed the STAR (Screening Test for Antibiotic Residues) method so that,

in addition to improving detection limits, there would be a homogeneous control in EU countries, thus avoiding the use of different methods or procedures (AFSSA, 1999).

4. Validation and accreditation of microbiological methods for detecting antibiotic residues

The implementation of quality systems as a result of the requirement established by Commission Decision 93/99/EC (EU, 1993b) and Royal Decree 1397/1995 (BOE, 1995) regarding the accreditation of official control laboratories in accordance with EN 45001-UNE 66-501-91 (UNE, 1991) (currently under the ISO/IEC 17025 Standard), together with the need to know the detection limits of the methods led to significant validation exercise of these methods.

The accreditation of the CNA by the Spanish National Accreditation Body (ENAC) under the then in force ISO/IEC 45001 standard regarding four of the microbiological methods for detecting antibiotic residues (four-plate and STOP screening methods, post-screening method by multi-plate assay and screening method in milk by inhibiting *Bacillus stearothermophilus*) was obtained in 1999. The accreditation of this type of microbiological methods under the ISO/IEC 17025 standard remained within the CNA's scope of accreditation until 2017.

The methods in which a sample is not extracted but rather is applied whole and directly on the culture medium on a test plate, such as the five-plate screening method for example, carry significant challenges for estimating their detection limit due to the complexity of adding antibiotic standards to the sample in a representative way. A protocol was established in the CNA for estimating detection limits through the homogenisation and freezing of meat samples and the combination of paper discs with non-homogenised tissue discs. These two systems were included in the Guideline for the validation of the Community Reference Laboratories for residues in food (CRL, 2010) and the results obtained in the validation were published in 2012 (Díez et al., 2012b).

This validation work did not only impact the CNA but it could also be used by the official control regional laboratories. For this, three collaborative tests were organised in 2005, 2012 and 2013 with 38, 28 and 26 laboratories respectively, in which all participants had to analyse the samples sent by the National Reference Laboratory. This test made it possible to benefit from a shared validation and made it easier for participating laboratories to be able to accredit the five-plate screening method in accordance with the ISO/IEC 17025 standard.

5. Analysis of samples by means of microbiological methods for detecting antibiotic residues

In the early years there was a significant load for analysing samples sent for initial analysis from the export programme for meat to the United States, the Border Health Services or the PNIR itself. Subsequently the majority of samples received were then sent off to confirm the positive results obtained by the official control regional laboratories. Thus, 79 % of the samples received between 1990 and 1999 came from these laboratories. During this time the CNA analysed 4 272 samples by means of microbiological methods for antibiotics residue analysis, the majority coming from muscle (55 %) and kidney (30 %), over half of them belonging to sheep.

Albeit to a lesser extent than the samples of products of animal origin, the CNA also received feed and zoosanitary product samples taken on farms in order to confirm if the labelling corresponded to its contents. The concentrations of antibiotics in those cases may be extraordinarily high and cause contamination problems in the instrumental methods so occasionally, prior to identifying illegal substances having a hormonal action in one of these samples, an analysis by microbiological methods was done in order to check if it contained high concentrations of antibiotics.

Royal Decree 1749/98 (BOE, 1998) establishes a sampling system in triplicate in order to undertake the initial, contradictory and final confirmation analyses. The National Reference Laboratory is in charge of carrying out the final confirmation analysis and may not participate in the contradictory analysis. Although at the beginning of the PNIR's application some microbiological screening and post-screening analyses were done as part of the contradictory or final confirmation analysis, the existence of maximum residue limits implies that a positive result must be confirmed by instrumental methods from the initial analysis itself, given that these are what determine whether or not said maximum limits were exceeded.

The samples received in the CNA were analysed by both the five-plate screening method for detecting antibiotic residues and the post-screening method for identifying groups of antibiotics. Among the groups of antibiotics identified in positive samples, tetracyclines, which involved more than 85 % of the antibiotics identified between 1993 and 1999, stand out (Figure 2).

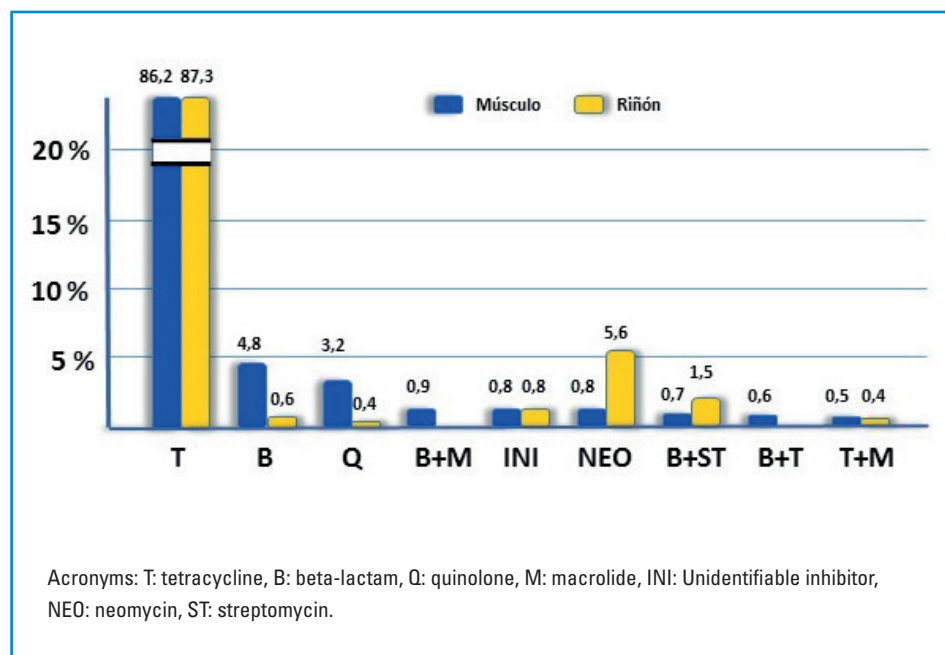


Figure 2. Groups of antibiotics detected in positive samples from all species.

In the case of sheep samples, more than 98 % of the positive samples were tetracyclines and, in accordance with the results from the physical-chemical confirmation methods, they mostly corresponded to chlortetracycline, for which the microbiological methods presented excess sensitivity, detecting quantities well below the established maximum residue limit. Although in other species the tetracycline residues were also the largest group, the aminoglycoside antibiotics also had a strong presence in positive bovine or pig kidney. On the other hand, quinolones were the most identified group in poultry and especially in fish positive samples.

The consistency between the screening and post-screening methods used was quite high, taking into consideration that the origin is different, European and American respectively, and they were not developed to be used as a part of the same analytical strategy. In 92 % of cases the results of both methods were positive or negative for the two, and the discrepancies were mainly due to negative results to the screening method that tested positive for tetracyclines in the post-screening. In addition to specific sensitivity differences between the methods, in the case of the kidney one possible explanation is that, as there is a difference in distribution of the antibiotics between the renal medulla and renal cortex, it is possible that an area with a lower or higher concentration than in the post-screening was used, because of the small amount of sample used in the screening. By homogenising a larger portion of the sample, the concentration is more representative (Figure 3).

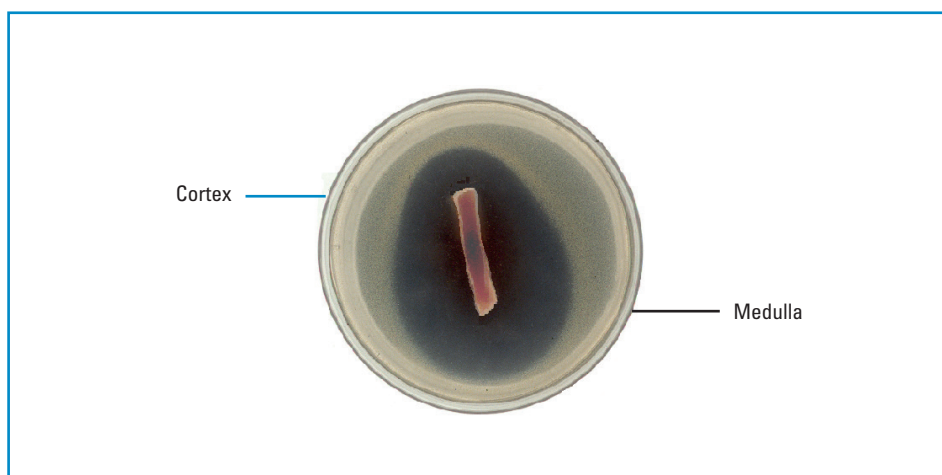


Figure 3. Differences in antibiotic accumulation in the cortex and medulla in a sheep kidney. Photo: G. Jiménez.

Within the Spanish National Residues Plan the level of identification of groups of antibiotics in positive samples for the screening method was very high. Between 1993 and 1999, in the Reference Laboratory, only 0.8 % of unidentifiable bacterial inhibitors were detected, compared to what happened in other programmes, with much higher percentages (Wilson et al., 1991) (Bergner-Lang et al., 1993).

6. Activities of Reference

6.1 Network of laboratories

At the national level, there were numerous laboratories involved in the control of antibiotic residues by microbiological methods given that these methods do not require sophisticated instrumentation and that there was a large number of samples to be analysed. More than 50 Public Health Laboratories and some other municipal and agricultural laboratories participated in the control of antibiotic residues.

At the international level, the CNEVA-LMV of Fougères (France) was appointed as the Community Reference Laboratory (CRL) for antibiotic residues in 1991 (EU, 1991). The Community Laboratory convened the first meeting of National Reference Laboratories in May 1994 and undertook its first visit to the CNA in July 1995. Since its appointment, it has held regular meetings with the National Reference Laboratories, some of which are specifically dedicated to microbiological methods. There has been frequent exchange of information, particularly with the National Reference Laboratories of Belgium, Denmark, Finland, the Netherlands, Italy and Portugal. In this last case there was also frequent contact with the research team from the University of Coimbra, which was reflected in a stay in the CNA and a joint publication (Pena et al., 2004).

In certain cases, stays were undertaken in some of these laboratories and furthermore, in the case of the United States, a training stay was completed in 1992 in the Antibiotic Residues Unit of the FSIS in Beltsville, Maryland and in the Microbiology Unit of the FSIS Laboratory in St. Louis, Missouri, in order to understand the analytical methods and strategies used in depth.

The duties of the National Reference Laboratories were initially established by Royal Decree 1262/1989:

1. Establish and coordinate the standards and methods of analysis for each residue or group of residues, insofar as corresponding official analysis methods are not established.
2. Organise periodic comparative tests on samples analysed in authorised monitoring laboratories.

Subsequently, Royal Decree 1749/1998 (BOE, 1998) established the new competencies and duties of the National Reference Laboratories:

- a. Coordination of the activities of the Official Control laboratories in charge of residue analysis and coordinating the standards and analysis methods of each type of residue or residue group in question.
- b. Collaboration with the competent authorities to organise a residue monitoring plan.
- c. Periodic organisation of comparative tests for each type of residue or residue group that have been assigned to the laboratory.
- d. Promoting and guaranteeing that the authorised monitoring laboratories respect the established detection limits.
- e. Ensuring the dissemination of the information supplied by the Community Reference Laboratories.
- f. Ensuring that it's possible for personnel to participate in developmental meetings organised by the Commission or the Community Reference Laboratories.
- g. Providing technical support and training to the staff of authorised monitoring laboratories.

6.2 Laboratory Communications

The FSIS central laboratories in the United States used a system of newsletters for their official control laboratories (*Laboratory Communications*) and in Spain, following this example, a similar system for communications between the National Reference Laboratory and the official control laboratories of the PNIR was implemented, largely from the regional governments (Autonomous Communities).

The first Laboratory Communication from the CNA was sent in 1991 and addressed how to avoid the false positive results in the detection of antibiotics by means of using a cellulose membrane applied to the four-plate method (CNA, 1991). In 1993 the information on physical-chemical methods of analysis of residues of products for zoonosanitary use was incorporated into the Communications system and, finally, in 2010 the Laboratory Communications system was generalised in order to provide information regarding all of the activities of reference assigned to the CNA. Between 1991 and 2018, 148 Laboratory Communications were sent regarding the microbiological methods for detecting antibiotic residues, 27 % of the 553-total sent by the CNA during this period.

6.3 Intercomparison exercises

One of the procedures for monitoring a laboratory's performance regarding ensuring the validity of its results described by the IEC/ISO 17025 standard (ISO, 2017) is comparing said results with those from other laboratories by means of proficiency tests or other interlaboratory comparisons.

In this context, between 1999 and 2014 the CAN, as a National Reference Laboratory, organised 15 intercomparison exercises for the five-plate screening method and 10 for the post-screening method by multi-plate assay in which official control laboratories could participate.

The number of participating laboratories was up to 47 in the case of the five-plate screening method and up to 10 in the post-screening method by multiple bioassay, which required a significant logistical effort.

In the case of the five-plate screening method, following the National Reference Laboratory of Denmark's example, antibiotic tablets commonly used in antimicrobial susceptibility testing were used as analytical samples. The goal of the comparison was to verify the method's sensitivity, so samples with homogeneous and stable antibiotic contents were required. These tablets, although they contain rather high levels of antibiotics for the residue methods, their advantage is that they are stable at room temperature, which makes their shipping via express mail easy, thus reducing expenses. Furthermore, the manufacturer was asked to also produce samples without antibiotics in order to be able to introduce blank samples in the exercises.

Regarding the analysis of the results, the model developed in the Netherlands for interlaboratory tests of the antibiotics susceptibility test on plates that includes the z-score estimation was followed (Mevius et al., 1994).

In the case of the post-screening method, the objective of the intercomparison exercises was to correctly identify the group of antibiotics present in the sent samples that, in this case, involved paper discs with antibiotics, dried and prepared in the reference laboratory itself, and which were sent to the participants via express mail.

In addition to these exercises, in 1997 an intercomparison exercise was organised for the microbial screening method of milk by inhibiting *G. stearotherophilus* intended for inter professional dairy laboratories.

The European Union Reference Laboratory also organised several intercomparison exercises for National Reference Laboratories, using milk samples or tissue samples from treated animals. In some occasions the test assessed the analytical strategy followed, which involved the instrumental confirmation and quantification techniques as well as the microbiological methods.

6.4 Collaborative tests

In order to provide control laboratories with the antibiotic residue five-plate screening method validation, and therefore enable the accreditation of the method in accordance with the IEC/ISO 17025 standard, three collaborative tests were organised.

In order to transfer the validation of this method from the National Reference Laboratory to control laboratories, two alternative ways might be followed:

On the one hand, participating in a collaborative test organised by the National Reference Laboratory, so that a shared (interlaboratory) validation of the procedure would be completed among all of the laboratories. Data obtained this way made up the validation of said test and, therefore, each official control laboratory could use them in order to evaluate the analytical method and determine if it was valid in its intended use, without requiring other verification tests.

On the other hand, those official control laboratories that did not participate in this interlaboratory validation could use the National Reference Laboratory's reference procedure with the terms of transfer derived from the tenets of the NT-55 technical note from the Spanish National Accreditation Body (ENAC, 2011). To that end, once the analytical procedure and validation data are transferred, the official control laboratory should verify the validation data reported by the CNA, completing a minimum of 10 tests per tissue or matrix for each antibiotic.

In the first collaborative test in 2005, 4 antibiotics were validated in muscle and 5 in kidneys. 38 laboratories from 17 Spanish regions participated. For 8 out of the 9 antibiotic residues (chlortetracycline, oxacillin, and ciprofloxacin in muscle and tetracycline, flumequine, neomycin, gentamicin and lincomycin in kidney) the percentage of false negative results was less than 5 % at the level of interest (maximum residue limit), meeting the criterion established by Commission Decision 2002/657/EC concerning screening methods (EU, 2002). Five laboratories did not detect benzylpenicillin and therefore, in this case, they did not meet the criterion about the level of false negative results not exceeding 5 %.

In the second collaborative test in 2012, 5 antibiotics were tested (chlortetracycline, amoxicillin, oxacillin, erythromycin and enrofloxacin) in muscle from different species (beef, sheep, pig meat and poultry). In this test, 28 laboratories from 15 regions participated. Four of the five antibiotics were detected at the level of the maximum residue limit, with a level of false negative results below 5 %. Only oxacillin had a higher level of false negative results, perhaps caused by incidences in the transportation of some samples.

In the third collaborative test in 2015, 6 antibiotics were tested (doxycycline, benzylpenicillin, cefotiofur, kanamycin, tilmicosin and marbofloxacin) in kidney, and furthermore, oxacillin in muscle. In

this exercise, 26 laboratories from 14 regions participated. All of the antibiotics were detected at the level of the maximum residue limit, with a level of false negative results below 5 %.

6.5 Conferences

In 1990, in order to facilitate the exchange of information with the network of official control laboratories, only a few months before Royal Decree 1262/1989, which started the PNIR, was published, the first conference was organised for the official control laboratories. In this first conference held on 27 and 28 June 1990, the microbiological screening and post-screening methods for antibiotic residues, as well as the investigation of *Listeria monocytogenes* in food were addressed (Figure 4).

The second conference on microbiological methods for antibiotic residues was held in 1994 which also included a presentation on chromatographic techniques given by Dr. Thea Reuvers, Head of the Zoonository Residues Unit. Subsequently conferences dedicated to microbiological methods for antibiotic residues were held in 1995, 1996, 1998, 2002, 2003 and 2006. Turn-out for the conferences was quite high: in 2003, for example, there were 85 attendees from 35 laboratories or other organisations at the conference dedicated to the microbiological methods for the control of antibiotic residues.

In addition to matters specific to the microbiological methods, other issues were discussed in the conferences, for example: legislation, control plans in Spain and the European Union, quality control and assurance in laboratories, safety in handling samples or resistance to antibiotics. Speakers from the Community Reference Laboratory and official Spanish control laboratories were also invited.

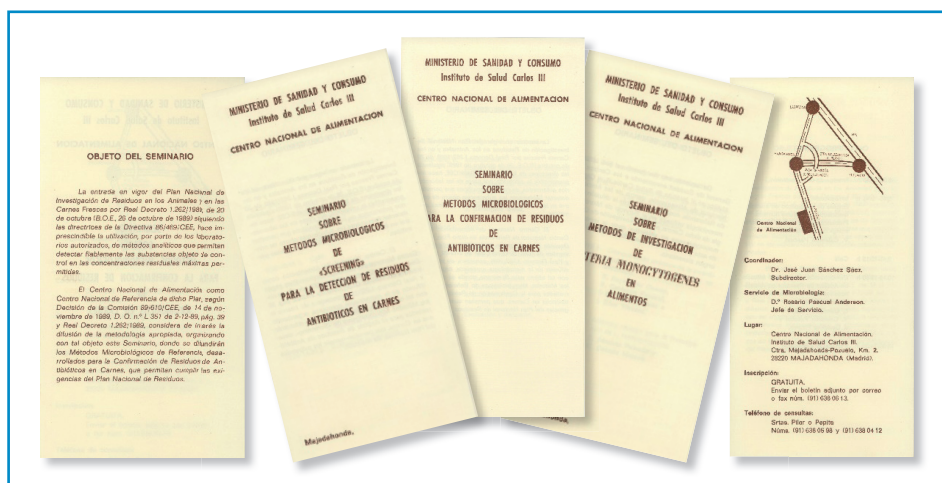


Figure 4. Programme from the first Landmark Conferences for antibiotic residues organised by the CNA's Microbiology Service in 1990, on antibiotic residues and *Listeria monocytogenes*.

Since 1998 the conferences were organised jointly between the Antibiotics and Zoonoses Residues Units. Since 2010, they have been held annually and were expanded to include all of the references and activities undertaken by the CNA, including those concerning antibiotic residues, in such a way that between 1990 and 2019, 29 landmark conferences have been held. As a matter of interest, one of the analysts who attended the first conference in 1990 was also in attendance at the one held in 2019.

6.6 Courses

From time to time analysts from the official control laboratories required practical training in the microbiological methods for the control of antibiotic residues. In this respect, in addition to facilitating stays in the laboratory, short courses were organised for groups of up to four analysts, ensuring that they were able to get the most out of the courses. Between 2002 and 2015, 17 of these courses on microbiological methods for determining antibiotic residues were organised.

Furthermore, the methods were shared in a variety of courses and conferences organised by other national bodies or foreign institutions.

6.7 Supplying materials

As part of their reference activities, the CNA took charge of providing the supply of certain materials to the official control laboratories.

In this respect, we highlight the suspensions of vegetative cells of bacterial strains used in microbiological methods. Some spore suspensions could be obtained from commercial sources, but others had to be prepared in the laboratory. However, the biggest problem was in the use of vegetative cells given that their culture and standardisation meant an excessive workload for the control laboratories. Therefore, by using freezing systems for the aforementioned bacterial suspensions (Reamer et al., 1995), these suspensions were prepared and standardised in the CNA (Díez et al., 1994) and were sent frozen at no charge to Spain's official control laboratories. From 2006 to 2014, 814 suspensions were provided, responding to 418 requests from official control laboratories. Most were *Kocuria rhizophila*, but there were also *E. coli*, *B. cereus*, *B. subtilis* and *S. epidermidis* suspensions.

In order to prevent false positive results in frozen pork kidney samples, it was necessary to place a cellulose membrane between the test plate cultures and the samples. A Spanish company provided a 20.7 µm thick transparent membrane that contained 70 % cellulose and 15 % plasticiser that's commonly used in food packaging and that was validated for its use in identifying antibiotic residues (Calderón et al., 1992a). Because the company did not wish to market the membrane, they provided CNA with enough so that it could be provided free of charge to the official control laboratories that requested it.

In addition to directly supplying materials, the availability of certain materials needed for carrying out the analyses, such as the test plate sensitivity control discs, was also supported by the CNA. These paper discs with antibiotics are similar to those commonly used in antimicrobial susceptibility testing, but the required antibiotic concentrations are lower and were not commercially available, so the control discs had to be prepared by the laboratories themselves. In order to make this material

more readily available and improve its consistency, the CNA was able to enlist a British company to produce them for the Spanish market.

6.8 Projects, publications, academic works and scientific information dissemination

Although the CNA's role was that of the reference laboratory and services provision as it regards to microbiological methods for the control of antibiotic residues, it also participated in research projects and undertook academic works, especially during its affiliation with the Carlos III Health Institute given its status as a public research organisation.

In this respect, it participated in three projects funded by the Health Research Fund and even presented a proposal, ultimately unfunded, to the Fifth EU Research Framework Programme for the development of microbiological screening and post-screening methods for antibiotic residues (STAR/POSTAR, 2000), in which 7 National Reference Laboratories from the European Union and the Community Reference Laboratory, coordinated by the CNA, all participated.

It also regularly participated in the Euroresidue conference in 1990, 1993, 1996, 2000 and 2004, in which it presented numerous written submissions and one oral communication (Calderón, 1996b).

Other laboratories or organisations from the regional governments also published the results of their control programmes (Gencat, 2015) or their laboratory analyses (Sánchez Cánovas et al., 1996) or comparisons of microbiological analysis methods for antibiotic residues (Marco et al., 1999).

As for academic works, a dissertation and two theses were presented in the Faculty of Pharmacy of the Complutense University of Madrid (Calderón, 2000b).

Regarding the scientific dissemination of the problem of antibiotic residue control and its monitoring, the CNA participated in two functions organised by the regional government of Madrid for the general public: receiving laboratory visitors during Science Week (2002) and setting up a fair stand about veterinary drug residues in food at the Madrid Science Fair (2001 and 2002) at the Trade Fair Institution of Madrid (IFEMA), which was attended by more than 50 000 people.

7. Current Situation

The microbiological methods for detecting antibiotic residues in foods of animal origin have played an important role in the National Residue Plans given that they have enabled the control of thousands of samples at a very low cost, a large and flexible processing capacity, adaptable to any number of samples and with less environmental impact than other methods.

The development of multi-residue physical-chemical instrumental methods, with detection limits better adjusted to the maximum antibiotic residue limits has resulted in a turning point and has limited the applicability of traditional microbiological methods for detecting antibiotic residues in the official control of the same.

In many European countries they have begun the transition from microbiological methods to instrumental ones, and some are using mixed strategies in which they apply microbiological methods in order to detect antibacterial residues, in addition to controlling specific groups of antibiotic residues by means of physical-chemical methods, that must subsequently be confirmed by other methods.

These analytical strategy changes may be assessed by comparing the number of positive samples and the type of antibiotic residues detected relative to those detected to date by means of microbiological methods.

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