Collaboration

Five-plate screening test for the detection of antibiotic residues

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Abstract

The five-plate screening test is a microbiological agar diffusion test for the detection of antibiotic residues in foods of animal origin and other matrices. Samples are assayed on test plates containing different microorganisms inoculated into culture media with different pH values. Antibacterial substances present in the samples diffuse around them leading to bacterial growth inhibition zones.

Key words

Antibiotic residues, bacterial inhibition, screening test.

Aim

The main aim of this technique is to detect antibacterial residues in foods of animal origin. The technique is a qualitative screening test for detecting bacterial growth inhibitors. A positive result will point to the presence of a bacterial growth inhibitor in the sample, whose nature and concentration level should be defined using more specialised techniques.

The technique is applied mainly to muscle and kidney samples, but it can be used for other matrices such as milk, eggs, feed, etc. In each case the usefulness of the technique should be assessed according to the intended use. For example, this technique is not suitable for reaching the required detection limits for sulphonamides in matrices such as muscle or kidney tissue but it can be useful for its detection in feed.

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Introduction

The antibiotics are used for treating various infectious diseases which affect food-producing animals. This practice is legal and widespread, requiring that treatment ceases before the animal is slaughtered or the production is collected, in order to avoid any residues which could harm the consumer. These residues can have toxic effects, essentially allergies, and they can affect the production of foods such as cheeses, yoghurt, or cold meats, where starter bacteria are part of the production process.

With the purpose of protecting the consumer and facilitating the free exchange of food products within the European Union, legislation exists which on the one hand sets maximum limits for residues through Regulation (EC) No. 470/2009 (EU,2009) and Regulation (EU) No. 37/2010 (EU, 2010), and, on the other hand, gives health control measures through Directive 96/23 (EU, 1996) and its transposition in Royal Decree 1749/98 (Real Decreto, 1998) which establishes the bases for the Spanish National Residue Monitoring Plan (PNIR).

The aim of the National Residue Monitoring Plan is to detect any illegal treatment and check whether the residue from veterinary medicinal products falls within the established maximum residue limits.

One of the specific characteristics of the sanitary control of antibiotic residues is the need to check whether the residue content in the food item exceeds the maximum limit established and is therefore potentially harmful to the consumer. It is not enough, therefore, to know if a given bacterial growth inhibitor is found or not in a food item; it must also be identified and quantified. Given that there is a need to monitor a great number of compounds in many different foods and samples, it becomes necessary to design an analytical strategy for analysing each residue or group of residues which can include the screening, post-screening, and confirmation phases. The relative non-specificity of the microbiological techniques means that their main use is found in the screening and post-screening phases prior to definite confirmation.

In general, the microbiological screening techniques based on the inhibition of bacterial growth used in the sanitary control of antibiotic residues are based on confronting a bacterial culture of a standardized strain, which is susceptible to certain antibiotics, with a portion or extract of the sample to be analysed. If the sample contains an antibiotic, this will inhibit the growth of the microorganism which can be observed through the formation of growth inhibition zones or changes in metabolic processes which impede, for instance, the acidification of the culture medium or the reduction of a colouring agent. This detection can be increased with the presence of products such as trimethoprim in the case of sulphonamides, with the use of susceptible microorganisms, or by adapting the pH of the culture medium to the optimum level for each antibiotic. When assay plates are being used, the presence of antibiotic residues is manifested through the formation of inhibition zones around the sample which can be measured for size.

The five-plate technique is the most commonly used microbiological screening test in Spain for the detection of antibiotic residues in foods of animal origin. It is based on the method published by R. Bogaerts and F. Wolf in 1980, which only used 4 plates, to which a fifth plate has been added, inoculated with *Escherichia coli*.

It is a cheap and simple screening multiresidue technique which allows a large number of samples to be analysed.

Materials and Method

1. Preparation of culture mediums

- Test agar for substances which inhibit pH 6 (Merck No. 10663 or equivalent): pH 6 ± 0.1
  Distilled water: 1 L
  Casein peptone: 3.45 g
  Meat peptone: 3.45 g
  NaCl: 5.1 g
  Agar: 13 g
  Autoclaving: 15 minutes, (121 ± 2) ºC

- Diagnostic Sensitivity Test (Oxoid No. CM261 or equivalent): pH 7.4 ± 0.1 (DST)
  Distilled water: 1 L
  Proteose peptone: 10 g
  Calf infusion solids: 10 g
  Glucose: 2 g
  Adenine sulphate: 0.01 g
  Guanine hydrochloride: 0.01 g
  Uracil: 0.01 g
  Sodium chloride: 3 g
  Xanthine: 0.01 g
  Disodium phosphate: 2 g
  Sodium acetate: 1 g
  Aneurine: 0.00002 g
  Agar: 12 g
  Autoclaving: 15 minutes, (121 ± 2) ºC

- Test agar for substances that inhibit pH8 (Merck No. 10664 or equivalent): pH 8 ± 0.1
  Distilled water: 1 L
  Casein peptone: 3.45 g
  Meat peptone: 3.45 g
  NaCl: 5.1 g
  Trisodium phosphate: 2.4 g
  Agar: 13 g
  Autoclaving: 15 minutes, (121 ± 2) ºC

2. Microorganisms

- *Bacillus subtilis* BGA (suspension of 10^7 spores/ml, Merck No. 10649, equivalent to CECT 482).
- *Kocuria rhizophila* CECT 241 (equivalent to ATCC 9341).
- *Escherichia coli* CECT 4201 (equivalent to ATCC 11303).

The spore suspensions are very stable whilst in refrigeration, but the same is not the case for vegetative cells, therefore they can be frozen in order to extend their lifespan and facilitate the preparation of test plates (Díez et al., 1994).

3. Reagent preparation

- Acetic acid 5%: 5 ml of acetic acid (Merck No. 62 or equivalent) diluted up to 100 ml with distilled water.
- Trimethoprim 30 µg/ml: Dissolve the trimethoprim standard dose in 5% acetic acid until reaching a 1,000 µg/ml solution and dilute with distilled water up to 30 µg/ml. The 30 µg/ml solution can be kept frozen for 6 months.

4. Standards

- 6 mm diameter antibiotic control discs:
  Benzylpenicillin 0.01 UI/disc (Mast PG0.01C or equivalent).
  Sulfadimidine 0.5 µg/disc (Mast SD0.5 C or equivalent).
  Streptomycin 0.5 µg/disc (Mast S0.5 C or equivalent).
  Ciprofloxacin 0.003 µg/disc (Mast CIP0.003 or equivalent).
- Antibiotics standards:
  Benzylpenicillin (Sigma PEN-NA or equivalent).
  Sulfadimidine (Sigma S 6256 or equivalent).
  Streptomycin (Sigma S 6501 or equivalent).
  Ciprofloxacin (Fluka 17850 or equivalent).

5. Plate preparation

Plate preparation with *B. subtilis* BGA

To prepare the plates with *B. subtilis* BGA (CECT 482), the microorganism (suspension of spores of *B. subtilis* BGA, 10^7 spores/ml) is inoculated into the pH 6, DST and pH 8 culture media so that a final concentration is achieved which is approximately 10^4 spores per millilitre of medium.

Normally this involves adding 100 µl of suspension for every 100 ml of medium, although it may be necessary to make adjustments in order to achieve the right inhibition zone size for the control disc.

In addition, a trimethoprim solution must be added to the DST plate in order to achieve a final concentration of 0.03 µg per ml of culture medium (100 µl of a 30 µg/ml solution of trimethoprim for every 100 ml of culture medium).

To prepare the plates with *K. rhizophila* CECT 241 (ATCC 9341) the microorganism in the pH 8 culture medium should be inoculated so that a final concentration of 10^4 colony forming units (cfu) per millilitre of medium is achieved.

To prepare the plates with *E. coli* CECT 4201 (ATCC 11303), the microorganism in the pH 8 culture medium should be inoculated so that a final concentration of 10^5 colony forming units (cfu) per millilitre of medium is achieved.

The amount of microorganism suspension to be added depends on its concentration and, as has already been mentioned, it may be necessary to make adjustments in order to achieve the right

inhibition zone size for the control disc. Frozen suspensions or recent microorganism cultures can be used.

The culture medium can be liquefied in the microwave and tempered in a water bath with a temperature no higher than 55 ºC. The inoculation can be done in a sterile flask or in the plate itself. It must be well shaken in order to ensure the microorganism is evenly spread out through the medium.

The culture medium is distributed on the plates such that a layer of approximately 2 mm thick is achieved. This means adding 10 ml in plates with a 90 mm diameter, 25 ml in plates with a 140 mm diameter, and 80 ml in 243 mm x 243 mm plates.

Lastly, it is left to solidify on a perfectly horizontal flat surface. The *K. rhizophila* and *E. coli* plates can be stored in the refrigerator for approximately 3 days, and the *B. subtilis* plates for 4 days.

Quality Control

As a form of quality control when preparing a series of plates, 6 mm diameter antibiotic or sulphamide discs are used. That way, in the centre of the *B. subtilis* BGA plate with an agar medium at pH 6, a disc with 0.01 UI of benzylpenicillin will be placed; in the *B. subtilis* BGA plate with DST medium a disc with 0.5 µg of sulphamide is used; and for the plates with an agar medium at pH 8, both *B. subtilis* BGA and *K. rhizophil*a, a disc with 0.5 µg of streptomycin is placed. Lastly, in the *E. coli* plates, discs with ciprofloxacin at 0.003 µg/disc are used.

These discs can be obtained commercially or they can be prepared in the laboratory. In case of the latter, 6 mm diamater paper discs should be soaked in 10 µl of the following solutions:

- PEN: Benzylpenicillin disc 0.01 UI/disc: 10 µl of a 1 Ul/ml solution.
- SDM: Sulphamide disc 0.5 µg/disc: 10 µl of a 50 µg/ml solution.
- STR: Streptomycin disc 0.5 µg/disc: 10 µl of a 50 µg/ml solution.
- CIP: Ciprofloxacin disc 0.003 µg/disc: 10 µl of a 0.3 µg/ml solution.

Once the antibacterial solution has been applied the next step is to use the disc in the corresponding plate, or desiccation, in order to preserve them for some time. After desiccation, the antibacterial discs are stored in the refrigerator. In order to desiccate them they are placed on a Petri plate marked with their symbol in an incubator or drying oven at 37 ºC for 30 minutes. In order to preserve them they will be placed in a sealed plastic tube with a stopper to ensure that humidity stays out.

The maximum period of conservation depends on the antibacterial solution and how it is stored. For example, for discs with the following antibacterial solutions, the approximate conservation periods in refrigeration are as follows:

- PEN: Benzylpenicillin 0.01 UI/disc: 2 weeks.
- SDM: Sulphamide 0.5 µg/disc: 1 month.
- STR: Streptomycin 0.5 µg/disc: 1 month.
- CIP: Ciprofloxacin 0.003 µg/disc: 1 month.

Collection of samples

In order to take samples from tissues (muscle, kidney) and aquaculture products, a sterile punch is used with an 8 mm internal diameter. Using this tool at least two different cylinders from two different parts of the previously frozen samples will be extracted.

The tissue cylinders are cut into 2 mm thick discs with a sterile scalpel. It will be necessary to change the punch and scalpel for each sample.

If the samples are feed samples, these should be mixed with sterile distilled water until a mass is formed. The amount of water to be added depends on the sample characteristics, therefore it is advisable to start with a 1:1 ratio and add more water until the sample achieves a doughy consistency.

Egg samples are homogenized in a paddle blender and then heated in a water bath at 70 ± 2 ºC for 20 minutes in order to deactivate the natural inhibitors.

Animal health products in powder form need to be mixed with a liquid so that they can be more easily applied on the plates. For this purpose, they are mixed with distilled water in a proportion which is sufficient to create a fluid which can be poured into the wells in the plates.

Analysis operations

Once the samples have been taken they are then placed on the testing plates. The 2 mm wide discs of tissue and aquaculture products samples will be placed in pairs in each of the five plates.

If the sample is pig kidney then a strip of cellulose membrane should be placed between the culture medium and the sample in order to avoid the natural inhibitors in the tissue (Calderón et al., 1992). Half of the sample will be placed over the medium, and the other half over the membrane.

For feed, milk and heat-treated egg samples and animal health products (in liquid or homogenized form), 8-mm diameter wells will be made and filled with the samples. The wells will be perforated until reaching the bottom of the culture medium, after it has solidified on the plate. In the case of milk it will also be necessary to apply *B. stearothermophilus* inhibition screening techniques.

Once the samples have been placed on the plates the incubation process can begin. The *B. subtilis* and *E. coli* plates are incubated at 30 ± 2 ºC and the *K. rhizophila* plates are incubated at 37 ± 2 ºC for 18 to 24 hours.

Processing of the results

After incubation checks are made on zones of microorganism growth inhibition in the area surrounding the samples and around the control discs. When the control discs show an inhibition zone at least 4 mm wide in the *K. rhizophila* plates and 6 mm for the others, it will be considered that the plates have worked as intended. The reading of the inhibition zone will be carried out more easily with a digital calibrator and a negatoscope as a light source; however a zone reader or even a millimetre ruler can be used. In any case, it is important that the measuring system is as standard as possible, so that measuring always takes place in the same conditions (Calderón et al., 1995).

The result of the test will be positive if zones of inhibition are at least (≥) 2 mm wide (measuring from the edge of the sample to the edge of the inhibition zone) in one or more plates. If this is not the case, the result is negative.

In the case of the frozen pig kidney, the reading of the inhibition zone will be carried out both for the zone where the sample is in direct contact with the culture medium as well as for the zone where it is in contact with the membrane. The latter will determine the final result.

The result is expressed as follows: the result of the analysis is positive or negative regarding the presence of bacterial growth inhibitors.

Even though it is not possible to identify the antibacterial agent in the sample using this technique, it is possible to compare the sizes of the inhibition zones obtained with the activity profiles for different studied antibiotics (Calderón, 2000). The different groups of antimicrobials show certain differences in terms of their activity profiles for the five plates used. Therefore the tetracyclines show the largest inhibition zones in the plate with a pH of 6; the beta-lactams show the same for the *K. rhizophila* plates and, in some cases, for the plate with a pH of 6; the aminoglycosides do the same for the plate with pH 8; and likewise the macrolides for the plate with *K. rhizophila*. The basic fluoroquinolones are similarly detected in the three *B. subtilis* plates, and their detection is improved for the *E. coli* plate. On the other hand, the rest of the quinolones cause the largest halos in the plates with pH 6.

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