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ABSTRACT

The STAR protocol is a Five Plate Test (FPT) developed several years ago at the Community Reference Laboratory (CRL) for the screening of antimicrobial residues in milk and muscle. This paper presents the validation of this method according to the European Decision 2002/657/EC and to an internal guideline for validation. A validation protocol based on “simulated tissues” and on a list of 16 representative antimicrobials to be validated has been implemented in our laboratory during several months for the STAR protocol. The performance characteristics of the method have been determined (specificity, detection capabilities CCβ, applicability, ruggedness). In conclusion, the STAR protocol is applicable to the broad spectrum detection of antibiotic residues in muscles of different animal species (pig, cattle, sheep, poultry). The method has good specificity (false positive rate 4%). The detection capabilities have been determined for 16 antibiotics from different families in relation to their respective Maximum Residue Limit (MRL): beta-lactams (penicillins and cephalosporins ≤ MRL), tetracyclines (≤MRL and ≤ 2.5 MRL), macrolides (2 MRL), quinolones (≤ 2 MRL), some sulphonamides (≤ 3 MRL), trimethoprim (2 MRL). However, the sensitivity of the STAR protocol towards aminoglycosides (> 8 MRL) and florfenicol (≤ 10 MRL) was unsatisfactory (>> MRL). The two objectives of this study have been met: firstly to validate the STAR protocol according to the European decision 2002/657/EC, then demonstrate that the validation guideline (Anonymous 2010) developed to implement this decision is applicable to microbiological plate tests even for muscle. The use of "simulated tissue appeared as a good compromise between spiked discs with antibiotic solutions and incurred tissues. In addition, the choice of a list of representative antibiotics allowed the reduction of the scope of the validation, which was already costly in time and effort.

Keywords: *Validation, decision 2002/657/EC, STAR protocol, screening, antimicrobial residues, muscle, plate test*

INTRODUCTION

Due to preventive or curative treatment of livestock, the presence of antibiotic residues could be found in food of animal origin. Traces of these antibiotics could cause various problems: problems of technological processing (e.g. milk), allergies to antibiotics following the ingestion of contaminated food (e.g. penicillins), antibiotic resistance of bacteria in humans, which could be transferred to pathogenic bacteria for humans. So, Maximum Residue Limits (MRLs) have been set for the antibiotics approved for use in veterinary medicine for livestock. The European Regulation No. 470/2009 of 6 May 2009 (EC 2009) establishes procedures for the establishment of Maximum Residue Limits of pharmacologically active substances in foodstuffs of animal origin and repeals Regulation No 2377/90 of 26 June 1990 (EC 1990). To monitor the presence of residues, the first step is the screening step which is to conclude whether a sample contains or not antibiotic residues at or above the MRL, in the case of permitted substances. Then, in case of positive screening it is necessary to use physico-chemical methods for the confirmation of identity and the quantification of the substance (EC 2002). The screening step is often based on microbiological screening methods that are cheap, easy to perform and do not need specific and expensive equipment. These methods are used since many years. Some of them have been developed even before the establishment of the Maximum Residue Limits (EC 1990) and many of them before the implementation of the rules for the validation of analytical methods (EC 2002).

The STAR protocol (for Screening Test for Antibiotic Residues), developed at the Community Reference Laboratory for antimicrobial residues in food (AFSSA Fougeres, France), is intended for the qualitative detection of residues of substances with antimicrobial activity in milk and muscle, using bacterial strains sensitive to antibiotics. This method is based on 5 different plates (Five Plate Test), dedicated to the detection of specific families of antibiotics. The first validation of the STAR protocol was organised in 1999 by the way of a collaborative study with spiked discs, blank muscles and incurred muscles (Fuselier et al. 2000). Seven antibiotics from 6 families have been tested. The detection levels were included between 1 MRL and 4 MRL, but it was dependent on the production of incurred materials. These results were promising for the implementation of the STAR protocol. Then the STAR protocol was validated for the screening of antibiotics in milk (Gaudin et al. 2004). The sensitivity was established by the analysis of milk samples spiked with 66 antibiotics at eight different concentrations. Ten different groups of antibiotics were studied: macrolides, aminoglycosides, cephalosporins, penicillins, quinolones, tetracyclines, sulphonamides, lincosamides, phenicols and miscellaneous drugs. The STAR protocol was able to detect 21

antibiotics at or below the maximum residue limit (MRL), and a further 27 drugs could be detected at levels from the MRL up to four times the MRL. The STAR protocol was at least twice as sensitive as conventional methods for macrolides, quinolones and tetracyclines. Each plate was preferentially sensitive for one or two families of antibacterials: the plate Bacillus cereus for tetracyclines, the plate Escherichia coli for quinolones, the plate Bacillus subtilis for aminoglycosides, the plate Kocuria rhizophila for macrolides and the plate Bacillus stearothermophilus for sulphonamides and beta-lactams.

Since 2002, every analytical method used for the analysis of monitoring routine samples has to be validated according to the European Decision 2002/657/EC (EC 2002) which concerns the performance of analytical methods and the interpretation of results. The level of validation of confirmatory methods is now quite satisfactory. However, very little information is contained in that decision concerning the validation of screening methods. Two main information are contained in the decision (EC 2002): firstly which performance characteristics have to be determined for a screening method and secondly the following information about the detection capability required for screening methods. Screening methods are “only those analytical techniques, for which it can be demonstrated in a documented traceable manner that they are validated and have a false compliant rate of < 5 % (β-error) at the level of interest shall be used for screening purposes in conformity with Directive 96/23/EC (EC 1996). In the case of a suspected non-compliant result, this result shall be confirmed by a confirmatory method.” Finally the difficulty is how to use this information to implement a validation for a screening method.

Moreover, the validation of microbiological screening methods presents specific issues, different from physico-chemical methods which are often specific methods. Firstly, the number of antibiotics to be validated is very high because all the antibiotics having a MRL in the corresponding matrix should be validated. It could be also the case for newly developed multi-residue methods by LC-MS/MS. However microbiological methods do not allow to identify the antibiotic residue present in the sample. So each antibiotic has to be tested independently and the number of analyses increased. Secondly, these kind of methods are only qualitative methods, giving a response as negative, positive or doubtful. Deciding on a sample size for qualitative inquiry can be even more difficult than quantitative because there are no definite rules to be followed. In general, sample size depends on the nature of the analysis to be performed, the desired precision of the estimates one wishes to achieve. The larger your sample size, the more sure you can be that their answers truly reflect the population. This indicates that for a given confidence level (e.g.. 95 %; β error = 5 %), the larger your sample size (n), the smaller your confidence interval (interval estimate of a

population parameter). For n=20 and a percentage of answers of 50% (i.e. 50 % answers positive; 50 % answers negative), the confidence interval is equal to 22 %. For n=60 and a percentage of answers of 50% (50 % positive; 50 % negative), the confidence interval is equal to 13 %. A greater sample is then required to decrease the confidence interval and therefore chance of error. Therefore from a statistical point of view, the number of samples to be analysed should be higher than for quantitative methods. Thirdly, spiking liquid matrices (e.g. milk, juice meat) is easy and validation could be performed on spiked samples. The problem is specific of solid matrices like muscle. In fact, plate tests are based on the analysis of raw muscle (slices of meat). Therefore the validation should be conducted on blank and incurred raw muscle. However, the production of incurred materials for each antimicrobials at one concentration would be time and money consuming. As a consequence, very few microbiological methods have been validated in the matrix, especially in muscle and other solid matrices according to the decision 2002/657/EC (EC 2002). A new guideline document supplements Commission Decision 2002/657/EC regarding the validation of screening methods. The Community Reference Laboratory (CRL) in Fougères in collaboration with the CRL in Berlin and in agreement with the CRL in Bilthoven, and after consultation through the NRL (National Reference Laboratories) network, has drafted this document with the purpose to assist residue laboratories to validate screening methods. This document is now finalised and officially published on the DGSANCO website since the 21st of January 2010 (Anonymous 2010). This guideline deals with the initial validation and also a shortened or 'abridged' validation, which under certain conditions, allows for the transfer of methods already validated in one laboratory to a second one. The guideline proposes some recommendations to implement a validation protocol for screening methods. It explains the performance characteristics to be determined (specificity, detection capability, robustness, …) and how to determine them in practice. Moreover, the number of samples necessary to validate a screening method is discussed. Finally, some new concepts have been introduced in the guideline: the preparation of “simulated tissues” and a list of representative substances to be validated. These two concepts will be detailed below in the validation protocol.

The validation conducted in this study is based on this validation guideline which is also the internal guideline in our laboratory for the validation of screening methods. Two objectives have been set: firstly to validate the STAR protocol for its application to the detection of antibiotic residues in muscles from different animal species according to the decision 2002/657/EC (EC 2002); secondly to show that the European guideline for the validation (Anonymous 2010) and based on the decision 2002/657/EC (EC 2002) was applicable to the validation of a microbiological screening method.

This paper will present the validation protocol and then the results of the validation of the STAR protocol for the screening of antibiotic residues in muscles of different animal species.

MATERIAL AND METHODS

Chemicals and standard solutions

Antibiotic and sulphonamide standards were provided by Sigma, except cefquinome (Intervet), ceftiofur (Upjohn), enrofloxacin (Bayer), doxycycline (Virbac).

STAR protocol

The STAR protocol is a Five Plate Test which was already published for the analysis of milk (Gaudin et al. 2004). Five test organisms (*Bacillus subtilis* B.G.A spores (MERCK), *Kocuria rhizophila* ATCC 9341 (Pasteur Institute, France), *Bacillus cereus* ATCC 11778 (Pasteur Institute, France), *Escherichia coli* ATCC 11303 (Pasteur Institute, France), *Bacillus stearothermophilus* ATCC 10149 (MERCK)) were inoculated in 5 different media. The 5 following culture media were used respectively: Antibiotic medium II at pH 8.0 (plate Bs8), Test agar at pH 8 (MERCK) (plate Kv8), Test agar at pH 6 (MERCK) (plate Bc6), Test agar at pH 8 (MERCK) (plate Ec8) and Diagnostic Sensitive Test (DST) (OXOID commercialised by UNIPATH LTD, Basingstoke, Hampshire, UK) (plate Bst). Culture media were prepared as recommended by the supplier and sterilised. Then 5 ml of inoculated medium were added on a Petri dish placed on a cold horizontal surface. In routine use, a cylindrical plug of 8 mm in diameter and 2 cm long is cut in frozen muscle using a cork borer. Then, slices of muscle samples of 2 mm in thickness are cut and put on the plates. The same protocol was applied to “simulated tissues”. Finally the plates are incubated: at 30°C for at least 18 hours for Bs8 and Bc6, at 37°C for at least 24 hours for Kv8, at 37°C for at least 18 hours for Bc6 and at 55°C for 15 to 16 hours for Bst.

A muscle sample was considered positive when the inhibition zone around meat sample was equal or superior to 2 mm in width on plates Bs 8, Kv 8, Bc 6 and Ec 8 and / or the inhibition zone equal or superior to 4 mm in width on plate Bst.

Since 2004, medium test agar pH 7.2 has been replaced by antibiotic medium II at pH 8.0 (Difco, reference 259310) because it was not commercialised anymore.. Moreover, the positive control of plate Bst was modified for practical reasons: sulfamethazine at 1000 µg l^{-1} has been replaced by amoxicillin at 40 µg l^{-1}. Positive controls consist of 30 µl of antibiotic solutions which are put on paper discs of 9 mm diameter (Durieux, France). There is a specific positive control for each plate: streptomycin at 2000 µg l^{-1} on plate Bs8, tylosin at 1000 µg l^{-1} on plate Kv8, oxytetracycline at 800 µg l^{-1} on plate Bc6, enrofloxacin 800 µg l^{-1} on

plate Ec8 and amoxicillin at 40 µg l^{-1} on plate Bst. The validity of each day of analysis depends on the results of the positive controls which have to be included in the following intervals: 5.5 ± 1.5 mm, 6.5 ± 1.5 mm, 6.0 ± 1.5 mm, 7.0 ± 1.5 mm and 6.0 ± 1.5 mm for Bs8, Kv8, Bc6, Ec8 and Bst respectively.

Validation protocol

Simulated tissues

In 2002, we have studied the sensitivity of 35 antibiotics by the way of antibiotic spiked discs. However, this way of working was not completely satisfying because the interference of the muscle matrix was absent. In fact, Okerman et al (1998a) showed that the tissue matrix has an effect on the sensitivity of the test plates. During that study, pieces of frozen meat laid on paper discs impregnated with antibiotic standard solutions have been used for the validation. Usually inhibition zones decreased when spiked meat samples have been analysed, compared to antibiotic spiked discs without meat. The same conclusions were reported by Pikkemaat et al. (2007). Because of the difficulty and the high cost of production of incurred materials for validation of a microbiological plate test, it was decided to work on what we called "simulated tissue. Furthermore, it was impossible to mix several antibiotics in the same sample, since the method was not specific and did not identify the molecule that produced an inhibitory effect. Moreover, one could observe a cumulative effect of antibiotics in their inhibitory activity when several antibiotics are contained in one sample.

Therefore, a preliminary study was conducted to determine what was the best way to prepare simulated tissues and what kind of preparation would give the closest result to the actual samples. This study was based on the experience of 2 National Reference Laboratories (Vicente Calderon, AESAN, Spain; Anna Liisa Myllyniemi, EVIRA, Finland) that had already worked on such samples. During this preliminary study, the way of preparation of simulated tissue was also tested for homogeneity of the material. Regarding the results, the homogeneity was satisfactory.

Production of spiked materials ("Simulated tissue")

A preliminary study has been conducted to determine the concentrations of antibiotics to be spiked in muscle samples for later validation. Sixteen different antibiotics have been tested at various concentrations (the MRL and according to the assumed detection limit at half MRL, 2 times the MRL or more) in simulated tissues. The tested concentrations were based on the results of the validation of the STAR protocol in milk (Gaudin et al. 2004). These tests were repeated several times. The concentration which always gave positive results (inhibition

zones higher than 2 mm, generally between 3 and 4 mm) and if possible concentrations lower than or equal to the respective MRLs have been chosen.

The antibiotics and the corresponding concentrations chosen for the validation are presented in Table 1.

Insert table 1 about here

Sixteen different antibiotics were used in this study. Nine different groups of antibiotics were studied : macrolides, aminoglycosides, cephalosporins, penicillins, quinolones, tetracyclines, sulphonamides, lincosamides and miscellaneous drugs. Stock solutions of the 16 different antimicrobials were prepared at a concentration of 1 mg ml^{-1}, after correction of potency. Then working solutions were prepared by dilutions in distilled water. Different batches of muscle were purchased in supermarkets. Muscle was firstly coarsely minced. To prevent antibiotic contamination, finely minced blank muscle samples were prepared first, the same day, with the 4 different batches of muscle, to be tested in parallel with the spiked samples. Then 1 ml of working solution was added to 100 g of blank minced muscle. Spiked muscle was homogenised during 15 minutes in the same rotary hatcher**.** At the end, the meat is finely minced. Each muscle material was finally put in plastic bottles or plastic bags. Each material was codified by the director of the study and then was frozen at -20°C. The "simulated tissues" were always analysed in a maximum period of 1 month.

Specificity

49 batches of pork muscle of different origins were analyzed in the end. Most of them (40 batches) have been tested in blind duplicate on 2 different days and by 2 different technicians.

Detection capabilities CCβ

According to EU Commission Decision 2002/657/EC (EC 2002), the detection capability (CCβ) of a method is defined as ''the smallest content of the substance that may be detected, identified and/or quantified in a sample with an error probability of β. In the case of substances with an established permitted limit, this means that the detection capability is the concentration at which the method is able to detect permitted limit concentrations with a statistical certainty of 1 – β.".

Given the expected detection capabilities for many antibiotics, which are often close to the MRLs, it was chosen to determine the detection capabilities by analyzing 60 minced muscle

samples spiked at one antibiotic concentration, instead of 20 samples to reach the most statically significant determination of the CCβ (EC 2002).

Insert Figure 1 about here

The preparation of the samples is detailed in figure 1. To introduce a maximum of variability in the determination of the detection capabilities, 30 samples have been prepared and analyzed by one technician and another 30 samples by other technician. In addition, the 30 samples have been divided into 2 sets of preparation. In each set, 15 samples for the same antibiotic concentration have been prepared and analyzed by each technician. For each antibiotic and each technician, the 2 sets have been spaced out, to introduce variability in the period of analysis (ambient conditions) and therefore variability of batches (media, bacteria, antibiotic standard...). Moreover, in each set of preparation of 4 antibiotics, 4 different batches of porcine muscle have been used. Finally, on each day of preparation, 2 different working solutions have been prepared from the stock solution of antibiotic and used to spike the different batches of muscle. The coding of the samples has been performed by the head of the study, to ensure that the analyses would be performed blindly, and then frozen at -20 °C.

In the determination of specificity and detection capabilities, all samples have been analyzed on the 5 plates of the STAR protocol to check the specificity of the plates for the different families of antibiotics. Five days of analyses have been performed with the STAR protocol for each set of preparation and each technician. Each day, 14 samples have been analysed blindly (12 spiked samples and 2 blank samples). Therefore, all blank and spiked samples have been analyzed twice or 3 times, on 2 or 3 different days.

One wants to underline that the analysis of the 60 samples for each antimicrobial were performed step by step. When one false negative result or more was obtained after the analysis of 10 samples, either the validation was stopped at this concentration and started again with an increased concentration if it was of interest (e.g. near the MRL) or the validation went on with the same antibiotic and the same concentration because the concentration was already much higher than the concerned MRL.

Determination of CCβ: After the analysis of the 60 spiked samples, the concentration level, where only less than 5 % of false compliant results remains was the detection capability CCβ of the method (3 false compliant results maximum out of 60 spiked samples).

Applicability study

The STAR protocol is implemented in parallel with the Four Plate Test for the analysis of muscles from different animal species , during the routine analysis of field samples (cattle, sheep, poultry, ...) in National Monitoring Plans since at least 5 years. Then, the positive samples at the screening step are confirmed by a multi-residue LC-MS/MS method (data to be published). Therefore, we have a lot of experience about the applicability of the method to muscles from different animal species.

However, to complete this study, we decided to perform a study to determine the CCβ of some antibiotics in cattle, sheep and poultry muscles, antibiotics for which the CCβ were determined previously in porcine muscle. Therefore, the CCβ of 5 antimicrobials (penicillin G at 25 µg kg^{-1}, doxycycline at 100 µg kg^{-1}, erythromycin at 400 µg kg^{-1}, gentamicin at 6000 µg kg^{-1}, enrofloxacin at 200 µg kg^{-1}) have been determined with 20 samples from each of the 3 species over 5 days and have been compared with the CCβ calculated for porcine muscle. In the applicability study, only the specific plate (which has presented inhibition zones for pig muscle) was tested with the corresponding antibiotic.

The applicability would be proved if the CCβ determined for these species are similar to the CCβ determined for porcine muscle (the average inhibition zones obtained for each species on the specific plate should be similar: accepted deviation ± 25%). A variability of 25 % is usually accepted with microbiological methods (for positive controls or spiked muscles). Moreover, this range of variability was observed during the validation in porcine muscle. The specificity of the STAR protocol with other species has also been studied by the analysis of different blank muscle samples.

Ruggedness study

The ruggedness of the method has been partly evaluated during the validation study, since different batches of media, bacterial strains, antimicrobial standards have been used. In addition, 2 technicians have been involved in the study, analysing each half of the samples and the variability between technicians could be estimated.

In the decision 2002/657/EC (EC 2002), the ruggedness is defined as " the susceptibility of an analytical method to changes in experimental conditions […] under which the method can be applied as presented or with specified minor modifications". A ruggedness study based on an experimental design was carried out and thus allowed testing analytical parameters which were different from those tested during the first step of validation. A full factorial design was

chosen for the analysis of the effects and interactions of 4 independent factors (Renard et al. 1992). The design matrix is presented in Table 2.

Insert Table 2 about here

Factors which may influence the measurement results have been selected: bacteria concentration (A), medium quantity (B) in the plate, incubation time (C) and pre-incubation time at room temperature (D). These factors have been modified in an order of magnitude corresponding to the usual differences: factor A concentration in bacteria: ± 30%; factor B quantity of medium 5 ± 0.5 ml; factor C incubation time ± 10%; factor D pre-incubation time (period at room temperature before incubation in the incubator: 1hour of pre-incubation or no pre-incubation).

The ruggedness study has been focused on 6 different representative antimicrobials which were specifically detected on each of the 5 plates on muscle: penicillin G at 25 µg kg^{-1} and sulfadimethoxine at 300 µg kg^{-1} on plate Bst, doxycycline at 100 µg kg^{-1} on plate Bc6, erythromycin at 400 µg kg^{-1} on plate Kv8, gentamicin at 6000 µg kg^{-1} on plate Bs8, enrofloxacin at 200 µg kg^{-1} on plate Ec8. The chosen concentrations were equal to the detection capabilities determined in the first part of the validation study for each of these antibiotics, except for gentamicin. The samples were prepared from pig muscle only. All b²lank and spiked minced muscle samples were prepared the day before starting the ruggedness study and were frozen at – 20°C. The study was performed blindly (codified samples). Then each day of analysis (each run), 4 different blank materials and 4 different spiked materials per antibiotic were analysed.

RESULTS AND DISCUSSION

Specificity

Specificity and detection capabilities were determined for pig matrix because it is the most common species analyzed in the laboratory. 49 different batches of porcine muscle have been tested. Few variations have been observed. Of 176 analyses in total, only 7 gave false positive results (4%). The 7 false positive results were obtained with 5 different muscle batches (2 false positive results for 2 batches and 1 false positive result for each of 3 batches). All false positive results have appeared on the plate Bst, none on the other plates. The specificity of the STAR protocol for the detection of antimicrobial residues in porcine muscle was very satisfactory.

Detection capabilities

The results presented in table 3 are those obtained from 60 samples for each antibiotic at least one tested concentration.

Insert Table 3 about here

For some antibiotics, after 15 or 30 analyzes by one or two technicians, either it turned out that too many false negative results were obtained at the selected concentration or on the opposite the first tested concentration gave higher inhibition zones than anticipated, suggesting that the validation could be performed at a lower concentration. At these concentrations, less than 60 samples have been analyzed, however it was helpful for the determination of the detection capability.

Figure 2 is a graphical representation of the relationship between detection capabilities calculated for the 16 representative antibiotics and their respective MRLs.

Insert Figure 2 about here

This figure allows one to quickly visualize the difference between CCβ and MRLs. The respective MRLs (µg kg^{-1}) are represented in plain bars. The hatched bars represent the respective CCβ (µg kg^{-1}). Therefore, the highest is the hatched area, the least sensitive is the STAR protocol for the corresponding antibiotic. On the opposite, when plain zone and hatched zone are very near, the sensitivity of the method for the corresponding antibiotic is satisfactory. When the CCβ is higher than 1000 µg kg^{-1}, the value of the CCβ is written on the top of the corresponding bar.

As a conclusion, the detection capabilities of penicillin G, cloxacillin and doxycycline were equal to or lower than their respective MRLs. The detection capabilities of tylosin, sulfadimethoxine, oxytetracycline, trimethoprim, erythromycin, ceftiofur, enrofloxacin and cefquinome were between 1.5 and 3 times their respective MRLs. The detection capability of lincomycin was between 4 and 5 times its MRL. Finally, the detection capabilities of gentamicin (GTM) (> 40*MRL), dihydrostreptomycin (DHS) (> 8*MRL) and florfenicol (> 10*MRL), were much higher than their respective MRLs. The STAR protocol is not suitable for the detection of the 2 aminoglycosides and probably not for the detection of the entire family, given the activity profiles obtained during the validation in milk (Gaudin et al. 2004). In fact, these activity profiles showed that GTM and DHS were the 2 aminoglycosides which were detected at the lowest concentrations, especially GTM. The sensitivities for GTM in milk

(MRL = 100 µg $l^{-1)}$ and to DHS (MRL = 200 µg l^{-1}) were equal to 3 and 5 times the respective MRLs. The sensitivities determined in milk for the other aminoglycosides were as follows: neomycin 1500 µg l^{-1} (MRL = 1500 µg l^{-1}), streptomycin 1000 µg l^{-1} (MRL = 200 µg l^{-1}), kanamycin 1000 µg l^{-1} (MRL = 150 µg l^{-1}), spectinomycin 40000 µg l^{-1} (MRL = 300 µg l^{-1}), paromomycin 2000 µg l^{-1} (not authorised in milk), apramycin 4000 µg l^{-1} (not authorised in milk). Therefore, the sensitivities for GTM and DHS were much better in milk than in muscle.

During the determination of detection capabilities in porcine muscle, it was demonstrated that the STAR protocol could provide an help to confirm the identity of a family of molecules present in the sample. Table 4 shows the plates on which each antibiotic reacted preferentially. Some antibiotics (e.g. cefquinome, ceftiofur, doxycycline, enrofloxacin and florfenicol) caused inhibitions onto several plates at the tested concentrations (including CCβ).

Insert Table 4 about here

The results were obviously similar to the global orientation given in the STAR protocol: beta-lactams and sulphonamides were detected preferentially on plate Bst, tetracyclines on plate Bc6, quinolones on plate Ec8 and macrolides on plate Kv8. However, there were 2 exceptions. The aminoglycosides have been detected on plate Bst with the concentrations tested for the determination of CCβ, whereas for higher concentrations than CCβ, they were detected on plate Bs8 = Bs7.2). However, when the validation of the STAR protocol has been performed in milk, Bs8 was the specific plate for the detection of aminoglycosides. Secondly, tylosin was detected preferentially on Bst instead of Kv8 at this concentration, which had already been observed during the validation of the STAR protocol in milk. So if a positive result is reported on plate Bst, the confirmation should be directed onto beta-lactams, sulphonamides, but also tylosin and aminoglycosides.

Applicability study

Given the unsatisfactory results for aminoglycosides (>> MRL), an additional study was conducted on GTM and DHS to choose one of these antibiotics and the good concentration for applicability and ruggedness studies. This study determined that the detection capability of GTM was around 4000 µg kg^{-1} and DHS around 6000 µg kg^{-1}. Therefore, GTM at 6000 µg kg^{-1} has been chosen, a concentration higher than the estimated detection capability (4000 µg kg^{-1}) due to reading difficulties (Partial Inhibition Zone (PIZ) and regrowth in the inhibition zone).

Figure 3 represents the average inhibition zones on the 5 different plates (Bst, Bc6, Kv8, Ec8 and Bs8) for blank muscles of different animal species and muscles spiked with 5 different antibiotics (each one reacted specifically on one of the plates) : penicillin G at 25 µg kg^{-1} on Bst (figure 3a), doxycycline at 100 µg kg^{-1} on Bc6 (figure 3b), erythromycin at 400 µg kg^{-1} on Kv8 (figure 3c), enrofloxacin of 200 µg kg^{-1} on Ec8 (figure 3d) and gentamicin at 6000 µg kg^{-1} on Bs8 (figure 3e). The results for the porcine muscle were those obtained during the determination of CCβ for 4 plates (Bst, Bc6, Kv8, Ec8) and during the additional study on aminoglycosides for plate Bs8.

Insert Figure 3 about here

The overall average inter-species was equal to 9.0 ± 1.3 mm, 5.2 ± 1.0 mm, 5.2 ± 1.4 mm, 6.1 ± 1.1 mm and 5.4 ± 0.9 mm for Bst, Bc6, Kv8, Ec8 and Bs8 respectively. Whichever was the tested antibiotic and the plate, the discrimination between blank and spiked samples was very clear and easy, for each species. Moreover, the average inhibition zone (IZ) for cattle, sheep and poultry muscle was in the fixed interval of plus or minus 25% compared to the average IZ for porcine muscle for plates Bst, Bc6, Ec8 and Bs8. Concerning plate Kv8, the average inhibition zone (IZ) for cattle and sheep muscle was in the fixed interval of plus or minus 25% compared to the average IZ for porcine muscle. The poultry muscle was out of the interval for plate Kv8, but with an average inhibition zone of 33% higher than the porcine muscle, so the sensitivity was better in the poultry muscle.

Two blank samples of cattle, 2 sheep and 2 poultry were analyzed for each plate and the analyses were repeated for 5 days. Over 10 cattle tested, we got 2 false-positive results, 1 for sheep and 1 for poultry on Bst plate. The blank bovine and ovine samples gave IZ of 3.7 ± 0.7 and 3.3 ± 0.4 mm, higher than porcine (1.8 ± 1.4 mm) and poultry (2.0 ± 1.4 mm) blank samples. All blank samples gave non specific inhibition on the plate Bst, higher than for the other plates. That is why the positivity threshold of the plate Bst was set at 4 mm, while the positivity threshold for the other plates was set at 2 mm (Fuselier at al. 2000). No false-positive results were obtained on the plate, whatever the species, on the 4 other plates.

In conclusion, the STAR protocol is applicable for the screening of antibiotic residues in the muscle of the 4 major species: pig, cattle, sheep, poultry and by extension the muscle of minor species, whatever is the plate.

Ruggedness study

During the determination of specificity and detection capabilities of the 16 antimicrobials, lots of different media (different preparation and validity date), different preparations of bacteria, different batches of antibiotic standards have been used over a period of 9 months of testing. Looking at the quantitative results (inhibition zones) obtained by the 2 technicians on muscle samples, , the variability (standard deviations) was rather low, similar to or lower than the variability that is fixed for the positive controls of the method. During this period, the positive controls were included in the intervals set in the STAR protocol. This is the first track to conclude that the STAR method is robust.

The average inhibition zones and the standard deviations obtained during the ruggedness study and during the first part of the validation (determination of CCβ) have been compared and are presented in Table 5.

Insert Table 5 about here

When the standard deviation in ruggedness conditions (SDi) is significantly larger than the standard deviation of the method in terms of reproducibility, the conclusion is predictable that all factors taken together have an impact on the results, even if no single factor has significant influence. In this case, the method is not sufficiently robust regarding the range of tested variations. From a quantitative point of view, we did not observe significant differences between the SD in ruggedness conditions and the SD during the first part of the validation for the 6 tested antibiotics. Therefore, the STAR protocol seemed to be robust for the 5 plates regarding the range of tested variations.

If we look at the mean inhibition zones obtained at each day (run) during the ruggedness study to the mean inhibition zones obtained during the determination of detection capabilities, it seemed that the sensitivity is very near, even if the average inhibition zones were often lower during the ruggedness study. In fact, the average inhibition zone of SDMX on plate Bst was significantly lower during the ruggedness study (Table 5). Therefore some of the tested factors had an effect on the sensitivity. Sulphonamides tested concentrations often gave Partial Inhibition Zones (PIZ), which are sometimes quite tricky to read for someone not trained. On the opposite, beta-lactams usually gave clear inhibition zones. So, slight variations like in the ruggedness study, even for a trained people, led to more effect on these PIZ than on clear inhibition zones. Therefore, it is logical that the Bst plate is less robust for the detection of SDMX than for penicillin G.

The ruggedness of the STAR method has been evaluated using an experimental design. The influence of the 4 factors (concentration of bacteria, quantity of medium, incubation time and pre-incubation time) on the mean inhibition zone (quantitative result), repeatability (coefficient of variation CV%) and false positive and false negative rate (qualitative result), as well as the interactions between factors, have been evaluated (Table 2). The responses for each plate for each run (day) were indicated (mean inhibition zone (IZ), the coefficient of variation (CV %) on the inhibition zone and false positive and false negative rates when influence was observed). From these results, the exploitation of the experimental design has been performed. The results are presented in Table 6 for the 6 antibiotics and the 5 plates.

Insert Table 6 about here

In conclusion, whatever the plates, there was no significant effect of analytical factors tested on the qualitative results of the STAR method (no influence on the false-positive rate and false-negative rate), except for the plate Bst with SDMX (**Table 6**). When testing SDMX, on day 4, the 4 tested samples gave false-negative results (**Table 7**). Therefore it has a strong impact of the combination of factors tested that day: A +, B +, C-, D-, i.e. increasing the concentration of bacteria, increasing the amount of medium, reducing the incubation period and without pre-incubation. In the optimization of microbiological methods, it is well known that increasing the concentration of bacteria or increasing the amount of medium may decrease the sensitivity of the plate. Moreover, as seen with other plates, a decrease of the incubation period can indeed reduce the sensitivity. It is also logical that a lack of pre-incubation may decrease the sensitivity of the plate. It was observed in fact that an increase of factors A and B may increase the false negative rate, while an increase of the incubation time and pre-incubation of one hour may increase the sensitivity. It is noticeable that 1 hour of pre-incubation at room temperature increased the mean inhibition zone of SDMX of 17%.

Insert Table 7 about here

Regarding the classical variability of results of microbiological plate tests, none of the factors or combination of factors had significant effect on the mean inhibition zone (quantitative result) for any of the tested antibiotics on the 5 plates. Finally, no significant effect has been observed on the repeatability of the method. Moreover, slights changes in the coefficient of variation never had effect on the qualitative results of the test for the tested antibiotics.
Therefore, the STAR protocol is a robust method for the detection of antibiotic residues in muscle. Some recommendations are given in the STAR protocol, concerning the different incubation periods for the 5 plates, because incubation time was already known as a critical

parameter. In the range recommended in the protocol, the method is robust. It should be noticed that increasing the incubation time for plate Kv8 increased the sensitivity of the plate of 13 % (**Table 6**). This is the reason why a longer incubation time (at least 24 hours) compared to the other plates is recommended in the STAR protocol. However, even at the lowest incubation time (21 hours), the results were satisfactory.

Finally, it has been demonstrated that a pre-incubation of 1 hour at room temperature could have a positive effect on the sensitivity of the test (plates Ec8, Bst (SDMX), Kv8) or no effect (plates Bc6, Bs8, Bst (penicillin G)), compared to no pre-incubation. However, pre-incubation is not a critical parameter because if people does not apply a pre-incubation, the results would be satisfactory and if pre-incubation is performed, the results would be equal or better. A recommendation of pre-incubation 1 hour at room temperature, should be added in the next version of the STAR protocol. Moreover, every day of analysis, specific positive control antibiotic paper discs are put on each plate. The results of these positive controls should be included in the range given in the STAR protocol. If it is the case, the results are valid.

Discussion

At this time, very few laboratories tried to validate their screening methods (microbiological or immunological methods) according to the decision 2002/657/EC (EC 2002) for 2 main reasons. Firstly, the decision 2002/657/EC (EC 2002) gave very little information and no technical recommendations for the implementation of the validation of a screening method. Secondly, especially for the validation of microbiological plate tests, the validation requires a long work, is time-consuming and quite expensive. In our laboratory, which is the Community Reference Laboratory for antibiotic residues, the validation of screening methods has been a subject of interest for many years. At this time, the validation of immunological tests and microbiological tests (tube tests and plate test) have been performed in our laboratory according to the European decision 2002/657/EC (Gaudin et al. 2004; Gaudin et al. 2007; Gaudin et al. 2009a; Gaudin et al. 2009b). The former “detection limit” has been replaced by a new performance characteristic called “detection capability CCβ”. To our knowledge, the other validation studies of microbiological plate tests according to the European decision (EC 2002) were from a National Reference Laboratory in the Netherlands (Pikkemaat et al. 2007; Pikkemaat et al. 2008; Pikkemaat et al. 2009b).

Regarding the time needed for this validation study, it would have been impossible to validate the STAR protocol for all the antibiotics having a MRL in muscle matrix (more than 50 antibiotics). The work of validation would be too long, expensive and laborious. Therefore

the recommendation to use a list of representative antibiotics was a very good compromise and allowed to reduce drastically the scope of the validation. It has been proposed since the first version of the European guideline for the validation of screening method in 2005 to validate a wide range test only for a list of representative antibiotics. It was adopted in the final version of the guideline (Anonymous 2010). The choice of the representative antibiotics is not fixed and is dependant of different factors: the activity patterns of different antibiotics in one family, the matrix, the use of antibiotics in one specific country, the assumed sensitivity of the method towards some antibiotics. The first step is to conduct a preliminary study that should allow to determine a common pattern of activity for one family or at least several substances of the family on a specific class of bacteria. Therefore one compound could be chosen to be representative of the other substances of the family in term of activity profile on bacteria. Then different antibiotics should be chosen for example for milk or muscle matrices. In fact the antibiotics used for intra-mammary treatment or for oral use could be different. Therefore, the interest of one antibiotic only used as intra-mammary treatment is very limited for a validation in muscle. Furthermore, if some antimicrobials are not used or not registered in some countries, there is no interest to validate for this compound if the method is intended to a national control. Finally, the selected analytes are dependent of the analytical method. So if the method to validate clearly badly detect one of antimicrobial, it is needless to determine its detection capability because this antibiotic would not be included in the scope of the method.

A similar proposition of validating for a list of representative antibiotics was made by Pikkemaat et al. (2009b). After the determination of the activity profiles of 36 antibiotics for the NAT-post-screening test for the detection of antibiotic residues in kidney, the authors suggested that the validation could be performed on a list of representative antibiotics to reduce the scope of validation for routine field laboratories for example.

One list of representative antibiotic was initially included as an example in the guideline which was extracted from the validation of the STAR protocol in milk (Gaudin et al. 2004). In fact, activity patterns have been determined for 66 antimicrobials having MRL in milk. The conclusion was that several antibiotics in one family could be gathered into one group because they showed similar activity profiles (same specific plate, similar sensitivity). Each family could be divided into 2 or more groups. Finally, 1 or 2 antibiotics per family have been chosen because its (or their) activity pattern (s) was (were) representative of one (or 2) group(s) of antimicrobials in the same family. Compared to this list of representative antibiotics, some molecules were replaced by others during the validation study of the STAR protocol, for the following reasons:

- Some antibiotics are only used as intra-mammary treatment (e.g. cefalonium has been replaced by ceftiofur because ceftiofur is widely used in cattle and swine to treat respiratory diseases while cefalonium is only administered intra-mammary), Moreover, it was determined during the validation of the STAR protocol in milk (Gaudin et al. 2004) that the least detected cephalosporins were cefquinome (MRL = 50 µg l^{-1}) and cefalexin (MRL = 200 µg l^{-1}). On the opposite, the best detected cephalosporins were cefazolin (Annex II for all tissues except milk) and cephapirin (MRL =50 µg l^{-1}). Finally, ceftiofur (MRL = 1000 µg l^{-1}) was better detected than cefquinome but less detected than cefazolin. Therefore, we have chosen to validate for ceftiofur and cefquinome because one was the least detected antibiotic of the family, with a low MRL (cefquinome) and the other (ceftiofur) has an intermediary detection with a high MRL. Regarding the activity profiles on the STAR protocol, it was assumed that if cefquinome is detected at 2000 µg kg^{-1} (CCbeta), cefazolin and cephapirin would be easily detected.

- The antibiotic should be largely used in the country of implementation of the method (e.g. in France, sulfamethazine replaced sulfathiazole). Moreover, colistin which belongs to the polymyxin family has been replaced by a second macrolide: tylosin because macrolides are often used for animal treatment (cattle, swine, poultry).

- During the development of the STAR protocol, it was already shown that the method had a very poor sensitivity for some antibiotics (e.g. flumequine, sulfaguanidine, colistin and spectinomycin), largely above the respective MRLs. So dihydrostreptomycin (DHS) replaced spectinomycin (level of detection in milk: 20000 to 40000 µg l^{-1}). During the validation of the STAR protocol in milk (Gaudin et al. 2004), the level of detection of colistin was included between 200 and 2000 times its MRL (MRL 50 µg l^{-1} in milk and 150 µg l^{-1} in muscle). Therefore the STAR protocol is not fitted to the detection of colistin or of spectinomycin.

- Some antibiotics are frequently detected in routine monitoring samples: the tetracyclines, particularly oxytetracycline (OTC). Thus, tetracycline has been replaced by oxytetracycline and doxycycline was added instead of flumequine. During the validation of the STAR protocol in milk, the limits of detection for OTC and TTC were both equal to 250 µg l^{-1} (Gaudin et al. 2004). The detection limits of doxycyline (50 µg l^{-1}) and CTC (75 µg l^{-1}) were better. Therefore tetracycline family has been divided in 2 groups from which OTC (the least detected) and doxycycline (the best detected) were chosen as representative compounds.

In a similar way, penicillin and cloxacillin have been chosen as the representative compound for the penicillin family because penicillin G (LOD 5 µg l^{-1}) was the best detected penicillin during the validation of the STAR protocol in milk and cloxacillin (LOD 60 µg l^{-1}) was the least detected penicillin (Gaudin et al. 2004). Moreover they belong to 2 groups of MRL (penicillin G 50 µg kg^{-1} in muscle and cloxacillin 300 µg kg^{-1}). Similarly, erythromycin (LOD 30 µg l^{-1}) and tilmicosin (LOD 50 µg l^{-1}) were the best detected macrolides in milk. On the contrary, tylosin (LOD 200 µg l^{-1}) was the least detected and was representative of the group of spiramycin (LOD 300 µg l^{-1}) and neospiramycin (LOD 200 µg l^{-1}). Moreover they also belongs to 2 groups of MRL (tylosin 100 µg kg^{-1} and erythromycin 200 µg kg^{-1}). Concerning quinolones, enrofloxacin was considered as representative of the quinolones family, except flumequine. In fact, flumequine is usually badly detected by many microbiological methods. When the STAR protocol was validated in milk, we have observed that enrofloxacin (detection limit = 20 µg l^{-1}), ciprofloxacin (10 µg l^{-1}), marbofloxacin (30 µg l^{-1}) and danofloxacin (15 µg l^{-1}) had the same activity profiles, with similar sensitivities (Gaudin et al. 2004). The quinolones which was badly detected was flumequine (> 600 µg l^{-1}). During the validation in milk, sulfanilamide, sulfapyridine, sulfadoxine, sulfacetamide, sulfaquanidine were the least sensitive sulphonamides (3.5 to 20 times the MRL (MRL = 100 µg/l^{-1}) and the most sensitive sulphonamides were sulfaphenazole, sulfathiazole, sulfachloropyridazine (MRL). The limits of detection of sulfadimethoxine (SDMX) (1.75 times the MRL) and sulfamethazine (2.5 times the MRL) were in between. The choice of sulphonamide was not based on the least detected compounds because it was too far from the MRL but was focused on 2 antibiotics which had intermediary sensitivities. Moreover these 2 sulphonamides are commonly used for animal treatment. Concerning the aminoglycosides, gentamicin in milk was the best detected antibiotic (limit of detection 300 µg l^{-1}) and spectinomycin was the least detected aminoglycoside (20000 to 40000 µg l^{-1}). The limit of detection for neomycin, kanamycin, streptomycin and DHS was equal to 1000 µg l^{-1}. Therefore DHS is representative of the 3 other aminoglycosides. Lincomycin was detected at 350 µg l^{-1} and pirlimycin at 100 µg l^{-1}. Therefore lincomycin was the least detected and is representative of the detection of lincosamides.

The specificity of the STAR protocol for the detection of antimicrobial residues in porcine muscle was very satisfactory. A similar result was obtained by Pikkemaat et al. (2009a) when implementing the STAR protocol on routine monitoring muscle samples in a comparative study. Only 1 % of false positive results were observed (6/591), on plate Bst, after the analyses of 591 routine monitoring samples, when the cut-off was set at 4 mm, which is the recommended cut-off in the STAR protocol for the plate Bst.

The results were obviously similar to the global orientation given in the STAR protocol: beta-lactams and sulphonamides were detected preferentially on plate Bst, tetracyclines on plate Bc6, quinolones on plate Ec8 and macrolides on plate Kv8. However, there were 2 exceptions. The aminoglycosides have been detected on plate Bst with the concentrations tested for the determination of CCβ, whereas for higher concentrations than CCβ, they were detected on plate Bs8 (= Bs7.2). However, when the validation of the STAR protocol has been performed in milk, Bs8 was the specific plate for the detection of aminoglycosides. Secondly, tylosin was detected preferentially on Bst instead of Kv8 at this concentration, which had already been observed during the validation of the STAR protocol in milk. So if a positive result is reported on plate Bst, the confirmation should be directed onto beta-lactams, sulphonamides, but also tylosin and aminoglycosides. A comparative study of 3 microbial screening tests including the STAR protocol applied to routine monitoring samples (Pikkemaat et al. 2009a) has similarly, shown that a macrolide (tulathromycin) was preferentially detected on plate Bc6 and Bst instead of plate Kv8. Therefore if no tetracyclines could be confirmed in a positive sample on plate Bc6, the confirmation could be directed towards tulathromycin. During this comparative study, the STAR protocol was able to detect the 4 MRL samples which contained antibiotic concentrations higher than their respective MRLs. Three of them were tetracyclines, detected preferentially on plate Bc6 and the remaining residue was sulfadiazine at 172 µg kg^{-1}. No information were available for beta-lactams, macrolides and quinolones because none of these families were found in the routine monitoring samples. The NAT-screening test was able to detect 4 samples containing aminoglycosides, but not the STAR protocol because this method is not enough sensitive towards the aminoglycoside family. Furthermore, the NAT-screening test is applied to kidney, while the STAR protocol is recommended for muscle. Moreover, it is well known that aminoglycosides concentrated in kidney, while very low concentrations could be found in muscle. The muscle matrix is not a satisfactory matrix for the screening of aminoglycosides. The results of this comparative study are in accordance with our validation data.

The detection capabilities have been determined for 16 antibiotics from different families in relation to their respective Maximum Residue Limit (MRL). The levels of detection of beta-lactams (penicillins and cephalosporins ≤ MRL) were very satisfactory because even the least detected compound has been detected at or below MRL. Concerning tetracyclines, OTC (the least detected) could be detected at levels below 2.5 times the MRL and doxycycline (the best detected) at the MRL. The levels of detection of macrolides (2 MRL), quinolones (≤ 2 MRL) and trimethoprim (2 MRL) were also satisfactory even for the least detected compound. The levels of detection of one representative sulphonamide SDMX (≤ 3 MRL) were slightly higher than in milk, Finally, the sensitivity of the STAR protocol towards

aminoglycosides and florfenicol was not satisfactory (>>MRL). Therefore the interest of the plate Bs8 in the STAR protocol could be discussed. This plate should be improved for its sensitivity or should be replaced by another plate. Other possibility is to use a complementary method focused on aminoglycosides which could replace the use of this fifth plate.

The validation of microbiological plate test is also a complex issue because of the solid matrices like muscle and kidney. In fact, the ideal solution would be to produce incurred samples from animal treatment for all antibiotics having MRL in the corresponding matrix and to validate all these antibiotics. However this is extremely time consuming and expensive. Furthermore, it is really difficult to obtain exactly a target concentration after the treatment of animals. So there are different possibilities to implement the validation of a microbiological plate test.

The first option is to use spiked paper discs to determine the sensitivity of the method from antibiotic standard solutions (Koenen-Dierick et al. 1995; Calderon et al. 1996, Currie et al. 1998; Ferrini et al. 2006). The main advantages are that this solution is less expensive and quicker. The validation could be implemented for a wide list of antibiotic residues. However, matrix components could affect the detection capabilities of a method. Okerman et al. (1998a) showed that only tetracyclines and quinolones were similarly detected with or without tissue. However, the detection of beta-lactams was better with antibiotic spiked discs without tissue than with tissues. For some antibiotics, the difference was only observed at low concentrations. Therefore, the use of antibiotic spiked discs is not totally satisfactory.

The second possibility is to use monitoring routine samples (Okerman et al. 1998b; Pikkemaat et al. 2009a; Schneider et al. 2009). Routine samples from monitoring plans have been analyzed with different methods including the method to be validated (including one or several physico-chemical confirmatory methods). The first disadvantage is that the number of samples containing residues is unknown. Moreover, very powerful confirmatory methods are needed to confirm all the samples, preferably even negative results at the screening step to check the false negative rate of the screening test. Therefore it is also costly and time consuming. Finally only a narrow range of antibiotics (those which are most frequently used in the country) is encountered. Schneider et al. (2009) confirmed the presence of antibiotics in 29 samples, from which 23 belonged to the tetracycline family. This kind of validation led very interesting information on the method, its practicability in routine conditions and some information on its performance but the results are limited by the range of antibiotics and their concentrations.

Sometimes, the validation study combined spiked discs and analyses of incurred materials, resulting from animal treatment (Myllyniemi et al. 1999; Myllyniemi et al. 2001). In this case, the authors used the activity patterns of antibiotic standard solutions to identify antibiotic residues in incurred materials. Furthermore, the STAR protocol has been validated in our lab using spiked paper discs, plus analysing field routine samples. The interest of spiked discs is that many antibiotics at many concentrations could be tested but limitation is that no matrix effect could be observed. It is interesting to obtain preliminary data on the evaluation of a new method and activity profiles of many antibiotics. But we know that matrix will affect the detection capabilities of the method. Therefore, field samples are very interesting because they are incurred materials and matrix effect could be demonstrated. However the limitation is that it is not known in advance how many positive samples are in the panel of field samples, which antibiotics would be detected and at which concentrations. Moreover another limitation is that a little variety of antibiotic residues or families are usually found, depending on the matrix. Okerman et al. (2004) used artificially contaminated (spiked tissue fluid) as well as incurred samples for the comparative study of 4 screening methods for the detection of tetracyclines in muscle matrix. This approach is also interesting because matrix effect could be observed both with spiked tissue fluid and incurred tissue.

The third possibility is the use of “simulated tissues” as it is proposed in this paper. It seemed to be a good compromise between spiked discs and incurred tissues and the nearest preparation from intact muscles because matrix effect could be evaluated. For the validation of a microbial screening assay, Pikkemaat et al. (2007) used 2 different approaches to produce fortified tissues. Firstly spiked minced was heated and centrifuged to extract meat juice (supernatant) which was applied directly onto the plate. Secondly incurred tissues were transformed into powder (using liquid nitrogen and blending the meat) and meat juice was extracted. Therefore, it was possible to obtain accurate concentrations in the samples by mixing incurred and blank materials. One conclusion was that “the sensitivity of the assay for quinolones decreased 2 fold when matrix samples are analysed” by comparison with antibiotic standard solutions. In these conditions, matrix effect was taken into account. Moreover, these 2 approaches are fitted for this screening test because in routine, meat juice samples would be analysed also. However, that sample preparation differed a lot from the routine application of the STAR protocol which is based on pieces of raw muscles laid directly on the plates. Therefore, in our case, our “simulated tissues” appeared to be as near as possible from real muscle samples. The binding of antibiotics to the minced tissue is possible during spiking. In 2009, Pikkemaat et al validated the NAT-post-screening test for the detection of antibiotic residues in kidney by determining the detection capabilities of 36 antibiotics in porcine as well as in homogenised fortified kidney samples (Pikkemaat et al.

2009b). After centrifugation, the supernatant could was analysed. The same final treatment was applied to routine monitoring samples. This is another way of preparing simulated tissues.

In the decision EC/2002/657 (EC 2002), there is no recommendation concerning the kind of materials to be analysed (spiked or incurred matrix) during the validation. Therefore all of the previous validation studies could be in accordance with the European decision if the way of determining performance characteristics was respected. The most important thing is to keep as close as possible to the materials analysed in routine use by the concerned method. Now the guideline for validation of screening methods (Anonymous 2010) recommends the use of "simulated tissues" (spiked tissues) when it concerns solid matrices and when it is impossible to obtain incurred tissues. Moreover, the validation could be restricted to a list of representative antibiotics. The validation which have been performed in the past on field samples led very interesting information concerning the method. However this kind of validation does not fit to the decision EC/2002/657 and to the recommendations of the validation guideline (Anonymous 2010). In fact the number of antibiotics tested is always very restricted. Moreover the number of samples to be analysed to determine the detection capabilities of antibiotics is never respected.

As a conclusion, there is no ideal way of validating microbiological plate tests which would not be time and money consuming. Obviously, validation of these kind of methods needs a lot of efforts from the lab which would validate the protocol for the first time (initial validation). In the guideline for validation of screening methods (Anonymous 2010), one of the recommendations is to reduce the work of validation when the method is transferred to another laboratory which would want to implement it in routine conditions. In fact, the number of samples to be analysed is reduced. The performance characteristics (e.g. detection capabilities) determined in the transfer lab have to be compared to those determined during the initial validation for a selected range of antibiotics. Then the participation to proficiency testing studies could complete the validation dossier.

Conclusions

The two objectives of this validation study have been fulfilled. Firstly, the STAR protocol has been validated for muscle by determining performance characteristics (specificity, detection capabilities CCβ for 16 representative antimicrobials, applicability, ruggedness), according to the European decision 2002/657/EC (EC 2002). The STAR protocol is applicable to the broad spectrum detection of antibiotic residues in muscles of different animal species (pig, cattle, sheep, poultry.). The method has good specificity (false positive rate 4%). The

detection capabilities have been determined for 16 antibiotics from different families in relation to their respective Maximum Residue Limit (MRL): beta-lactams (penicillins and cephalosporins ≤ MRL), tetracyclines (≤MRL and ≤ 2.5 MRL), macrolides (2 MRL), quinolones (≤ 2 MRL), some sulphonamides (≤ 3 MRL), trimethoprim (2 MRL). However, the sensitivity of the STAR protocol towards aminoglycosides and florfenicol was not satisfactory (>>MRL). Finally, this study has shown that the STAR method is a robust screening method, insensitive to reasonable variations analytical parameters such as concentration of bacteria, amount of medium, incubation period and pre-incubation or not.

Secondly, the applicability of the European guideline (Anonymous 2010) to the validation of a microbiological screening method and for muscle has been demonstrated. The use of "simulated tissue" appeared to be a very good compromise between antibiotic spiked discs and incurred samples. Moreover, the choice of a list of representative antimicrobials was also very interesting to reduce the scope of validation.

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Figure 1. Preparation of the simulated tissues.

Two technicians prepared each 30 samples per antibiotic (AB), divided in 2 sets of 15 samples each (Set 1 and Set 2). The 2 sets have been spaced out, to introduce variability in the period of analysis (ambient conditions) and therefore variability of batches (media, bacteria, antibiotic standard...). The example of preparation of Set 1 by technician 1 is presented here. Four different batches of porcine muscle have been used. Two different working solutions (WS) have been prepared from the stock solution of antibiotic and used to spike the different batches of muscle. Finally 15 samples have been prepared per antibiotic.

Figure 2. Relationship between the calculated detection capabilities and the respective MRLs of the 16 representative antibiotics.

The 16 representative antibiotics are represented in x-axis. The calculated CCβ of the 16 tested representative antimicrobials and the respective MRLs are represented in y-axis. MRL = Maximum Residue Limit.

Figure 3. Results of the applicability study of the STAR protocol to muscles from different animal species, on the 5 different plates.

The results of the applicability study for the 5 plates are represented: 2a. penicillin G at 25 µg kg^{-1} on plate Bst, 2b. doxycycline at 100 µg kg^{-1} on Plate Bc6, 2c. erythromycin at 400 µg kg^{-1} on Plate Kv8, 2d. enrofloxacin of 200 µg kg^{-1} on Plate Ec8 and 2e. gentamicin at 6000 µg kg^{-1} on Plate Bs8. In x-axis, the 4 different animal species tested are represented. In y-axis, the average inhibition zone (in mm) on the 5 different plates obtained during the applicability study for bovine, ovine and poultry muscles and during the determination of CCβ for porcine muscle are represented for blank samples and spiked samples.

Figure 1.

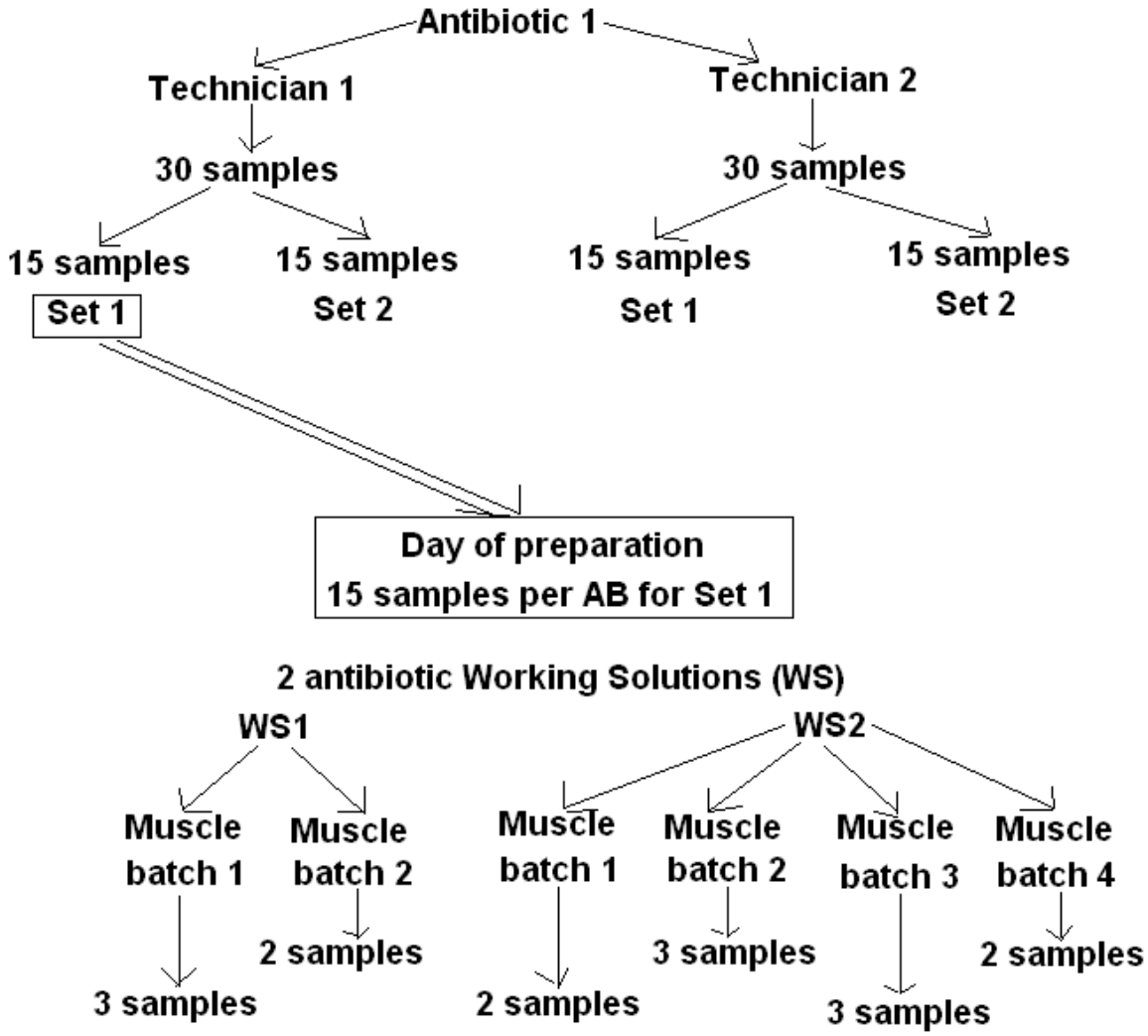

Figure 2.

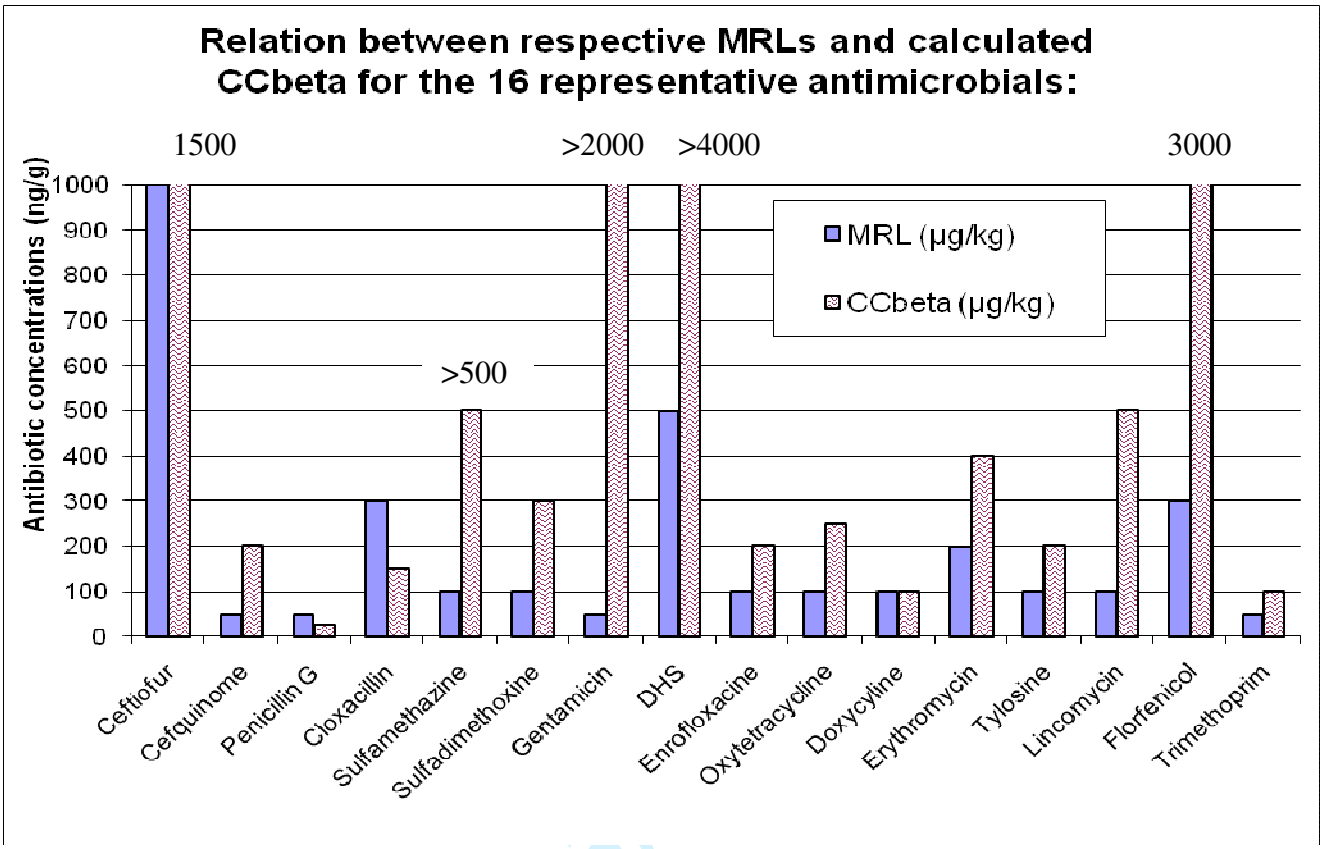

Figure 3.

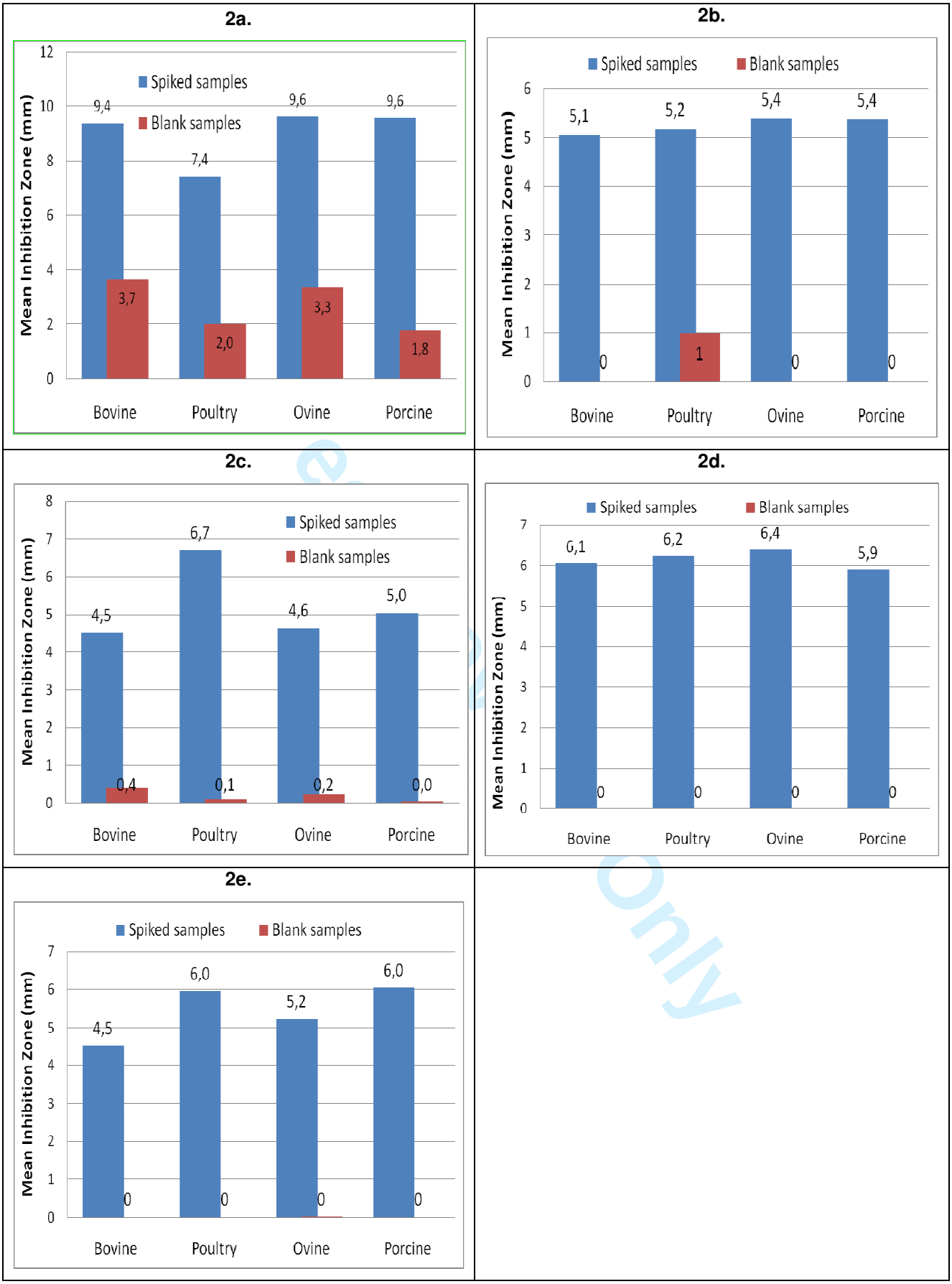
2a.
Spiked samples
Blank samples
Mean Inhibition Zone (mm)
9,4
3,7
7,4
2,0
9,6
3,3
9,6
1,8
Bovine
Poultry
Ovine
Porcine
2b.
Spiked samples
Blank samples
Mean Inhibition Zone (mm)
5,1
0
5,2
1
5,4
0
5,4
0
Bovine
Poultry
Ovine
Porcine
2c.
Spiked samples
Blank samples
Mean Inhibition Zone (mm)
4,5
0,4
6,7
0,1
4,6
0,2
5,0
0,0
Bovine
Poultry
Ovine
Porcine
2d.
Spiked samples
Blank samples
Mean Inhibition Zone (mm)
6,1
0
6,2
0
6,4
0
5,9
0
Bovine
Poultry
Ovine
Porcine
2e.
Spiked samples
Blank samples
Mean Inhibition Zone (mm)
4,5
0
6,0
0
5,2
0
6,0
0
Bovine
Poultry
Ovine
Porcine

Table 1. Chosen antibiotics and corresponding concentrations for the validation study.

Antibiotic families	Representative antibiotics	MRL (µg kg^{-1})	Chosen concentrations (µg kg^{-1})
CEPHALOSPORINS	*Ceftiofur*/Cefquinome	1000/50	1500/200
PENICILLINS	Penicillin G/Cloxacillin	50/300	25/150
SULFONAMIDES	*Sulfamethazine*/*Sulfadimethoxine*	100/100	500/300
AMINOGLYCOSIDES	Gentamicin (GTM) /*Dihydrostreptomycin (DHS)*	50/500	2000/4000
QUINOLONES	Enrofloxacin	100	200
TETRACYCLINES	*Oxytetracycline/Doxycycline*	100/100	250/100
MACROLIDES	Erythromycin/*Tylosin*	200/100	400/200
LINCOSAMIDES	Lincomycin	100	500
PHENICOLS	*Florfenicol*	300	3000
MISCELLANEOUS	Trimethoprim	50	100

The antibiotics in italics are the antibiotics which have been substituted to the antibiotics initially included in the list of representative antibiotics.

Table 2. Design matrixof the ruggedness study .

Run	Levels	A[a]	B[b]	C[c]	D=ABC[d]	AB+CD	AC+BD	BC+AD
1	+	-*	-	-	-	+	+	+
2	+	+	-	-	+	-	-	+
3	+	-	+	-	+	-	+	-
4	+	+	+	-	-	+	-	-
5	+	-	-	+	+	+	-	-
6	+	+	-	+	-	-	+	-
7	+	-	+	+	-	-	-	+
8	+	+	+	+	+	+	+	+

Run 1 = Day 1

[a] Concentration of bacteria; [b]Medium quantity; [c] Incubation time, [d] Pre-incubation time

** Each factor has been modified in an order of magnitude corresponding to the usual differences: factor A concentration in bacteria: ± 30%; factor B quantity of medium 5 ± 0.5 ml; factor C incubation time ± 10%; factor D pre-incubation time (period at room temperature before incubation in the incubator: 1hour of pre-incubation or no pre-incubation).*

+: increasing of the factor (i.e. + 30 %); -: decreasing of the factor (i.e. - 30 %)

AB+CD, AC+BD and BC+AD are the evaluation of the impact of the combination of the different factors

Table 3. Detection capabilities CCβ (µg kg^{-1}) for the 16 antimicrobials

Antibiotic family	Antibiotic	MRL muscle (µg kg^{-1})	Mean IZ * (mm) ± (SD)	Qualitative results	CCβ (µg kg^{-1})
PENICILLINS	Penicillin G	50	9.7 ± 1.1	60+	≤ 25
	Cloxacillin	300	6.4 ± 0.2	56+/4- (at 150 µg kg^{-1})	Between 150 and 300
CEPHALOSPORINS	Cefquinome	50	6.0 ± 0.3	14+/1D (at 100 µg kg^{-1}) 60+ (at 200 µg kg^{-1})	Between 100 and 200
	Ceftiofur	1000	5.3 ± 0.7	56+/2D/2-	1500
TETRACYCLINES	Oxytetracycline (OTC)	100	5.1 ± 0.5	60+	≤ 250
	Doxycycline	100	5.4 ± 0.5	60+	≤ 100
MACROLIDES	Erythromycin	200	5.0 ± 1.6	56+/1D/3-	400
	Tylosin	100	5.1 ± 0.7	55+/3D/2-	200
QUINOLONES	Enrofloxacin	100	5.9 ± 1.9	8+/4D/3- (at 100 µg kg^{-1}) 60+(at 200 µg kg^{-1})	Between 100 and 200
SULFONAMIDES	Sulfadimethoxine (SDMX)	100	8.8 ± 1.3	12+/1D/2- (at 200 µg kg^{-1}) 59+/1D(at 300 µg kg^{-1})	Between 200 and 300
	Sulfamethazine (SMZ)	100	5.4 ± 2.5	37+/2D/21-	> 500
AMINOGLYCOSIDES	Dihydrostreptomycin (DHS)	500	2.0 ± 0.7	24+/3D/33-	> 4000
	Gentamicin (GTM)	50	4.3 ± 0.4	45+/3D/12-	> 2000
LINCOSAMIDES	Lincomycin	100	5.8 ± 0.6	60+ (at 500 µg kg^{-1})	Between 350 and 500
MISCELLANEOUS	Trimethoprim (TMP)	50	7.9 ± 0.8	5+/10- (at 50 µg kg^{-1}) 59+ out of 59 (at 100 µg kg^{-1})	Between 50 and 100
	Florfenicol	300	4.8 ± 0.2	8+/3D/19- (at 1000 µg kg^{-1}) 60+(at 3000 µg kg^{-1})	Between 1000 and 3000

SD = Standard deviation; IZ: Inhibition zone (mm); +: positive result; -: negative result; D: doubtful result (0<ZI<2 mm).

Table 4. Specificity of the plates.

Antibiotic family	Antibiotic	Specific plate
PENICILLINS	Penicillin G	Bst
	Cloxacilline	Bst
CEPHALOSPORINES	Cefquinome	Kv8 (Bst and Ec8)
	Ceftiofur	Bst (and Ec8)
TETRACYCLINES	Oxytetracycline (OTC)	Bc6
	Doxycycline	Bc6 (and Bs8)
MACROLIDES	Erythromycin	Kv8
	Tylosin	Bst
QUINOLONES	Enrofloxacin	Ec8 (and Bst)
SULFONAMIDES	Sulfadimethoxine (SDMX)	Bst
	Sulfamethazine (SMZ)	Bst
AMINOGLYCOSIDES	Dihydrostreptomycin (DHS)	Bst
	Gentamicin (GTM)	Bst
LINCOSAMIDES	Lincomycin	Bst
MISCELLANEOUS	Trimethoprim (TMP)	Bst
	Florfenicol	Bs8 (Bc6 and Bst)

Table 5. Comparison of the mean Inhibition zones (IZ) and the standard deviations (SD) calculated during the determination of the CCβ and during the ruggedness study.

Antibiotic family	Antibiotic	Tested concentration (µg kg^{-1})	Plate	Mean IZ * (mm) ± SD CCβ	Mean IZ * (mm) ± SD Ruggedness
PENICILLINS	Penicillin G	25	Bst	9.7 + /- 0.2	8.5+/-1.2
SULFONAMIDES	Sulfadimethoxine (SDMX)	300	Bst	8.8 + /- 1.3	5.3+/-1.6
TETRACYCLINES	Doxycycline	100	Bc6	5.4 +/- 0.5	4.3+/-0.6
MACROLIDES	Erythromycin	400	Kv8	5.1 +/- 1.1	4.0+/-1.0
QUINOLONES	Enrofloxacin	200	Ec8	6.2 +/- 1.0	6.7+/-1.2
AMINOGLYCOSIDES	GTM	6000	Bs8	6,0+/-0,7	6.2+/-0.5

**IZ = Inhibition zone of the specific plate, mean of the values of the 4 sets (2 technicians)*

SD = Standard Deviation

Table 6. Exploitation of the factorial design : Influence of the 4 experimental factors on the detection of 6 tested antibiotics on the 5 plates of the STAR protocol.

		Factor				Interaction			Mean
Bc6	*Response*	*A*[a]	*B*[b]	*C*[c]	*D*[d]*=ABC*	*AB+CD*	*AC+BD*	*BC+AD*	*I*
	Mean IZ	-0.12	-0.01	-0.15	0.05	-0.13	0.01	-0.16	4.32
	CV (%)	-2.03	-3.08	5.23	2.75	2.91	-3.09	-1.88	11.21
Kv8	*Response*	*A*	*B*	*C*	*D=ABC*	*AB+CD*	*AC+BD*	*BC+AD*	*I*
	Mean IZ	-0.07	0.00	0.50*	0.28	0.12	-0.41	0.22	3.97
	CV (%)	-1.27	4.04	0.58	5.43	0.07	2.69	-1.93	16.07
Ec8	*Response*	*A*	*B*	*C*	*D=ABC*	*AB+CD*	*AC+BD*	*BC+AD*	*I*
	Mean IZ	-0.25	-0.58	-0.22	0.77	-0.45	-0.03	-0.33	6.72
	CV (%)	0.38	0.97	-0.07	-1.14	-0.47	-0.08	1.88	4.82
Bst	*Response*	*A*	*B*	*C*	*D=ABC*	*AB+CD*	*AC+BD*	*BC+AD*	*I*
SDMX	Mean IZ	0.34	-0.38	0.17	0.92	-0.30	-0.03	0.91	5.30
	CV	-0.26	0.15	-2.72	-0.01	0.25	-0.76	1.70	4.70
	False +	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00
	False -	0.50	0.50	-0.50	-0.50	0.50	-0.50	-0.50	0.50
Penicillin	Mean IZ	0.25	-0.20	-0.44	0.02	0.59	-0.42	0.03	8.55
	CV	-1.44	1.61	-2.91	1.74	-2.60	3.17	0.12	9.18
	False +	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00
	False -	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00
Bs8	*Response*	*A*	*B*	*C*	*D=ABC*	*AB+CD*	*AC+BD*	*BC+AD*	*I*
	Mean IZ	-0.11	0.00	0.03	0.02	-0.11	-0.23	0.00	6.16
	CV (%)	0.67	-0.84	0.65	0.53	0.01	0.14	0.81	6.07

[a] *Concentration of bacteria;* [b]*Medium quantity;* [c] *Incubation time,* [d] *Pre-incubation time*

*IZ = Inhibition zone 5MM°; CV: Coefficient of variation of the mean IZ (%); False +: False positive rate; False -: False negative rate. The mean false negative rate is equal to 0.50, that means 50 % of false negative results. * Increasing the incubation time (C) for plate Kv8 increased a little the sensitivity of the plate (13 %).*

Table 7. Ruggedness study for plate Bst: Design matrix and experimental design calculation.

Run	Mean IZ SDMX	CV	False +	False -	Mean IZ Penicillin	CV	False +	False -
1	4.8	17.5	0	0	9.1	10.9	0	0
2	8.0	18.0	0	0	9.3	10.3	0	0
3	4.7	13.9	0	0	7.5	22.5	0	0
4	3.0	15.4	0	100*	10.0	4.6	0	0
5	5.3	10.2	0	0	9.1	2.0	0	0
6	4.6	7.6	0	0	7.5	7.1	0	0
7	5.1	13.4	0	0	7.5	7.1	0	0
8	6.9	11.8	0	0	8.4	8.9	0	0

*IZ: Inhibition zone (mm); False + = False positive rate; False -: false negative rate; CV: Coefficient of variation (%) = (mean IZ)/(SD IZ)*100; SD: standard deviation.*

Run 1 = Day 1

**A strong impact of the combination of factors tested on day 4 was observed on false - rate: A +, B +, C-, D-, i.e. increasing the concentration of bacteria, increasing the amount of medium, reducing the incubation period and without pre-incubation.*