HAL
archives-ouvertes.fr

Validation of a wide spectrum microbiological tube test, the EXPLORER test, for the detection of antimicrobials in muscle from different animal species

Valerie Gaudin, Celine Hedou, Eric Verdon

► To cite this version:

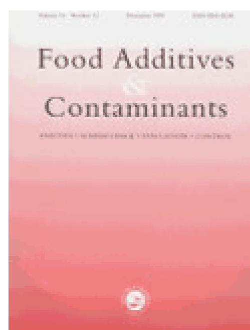
Valerie Gaudin, Celine Hedou, Eric Verdon. Validation of a wide spectrum microbiological tube test, the EXPLORER test, for the detection of antimicrobials in muscle from different animal species. Food Additives and Contaminants, 2009, 26 (08), pp.1162-1171. 10.1080/02652030902939671 . hal-00573896

HAL Id: hal-00573896
https://hal.archives-ouvertes.fr/hal-00573896

Submitted on 5 Mar 2011

HAL is a multi-disciplinary open access archive for the deposit and dissemination of scientific research documents, whether they are published or not. The documents may come from teaching and research institutions in France or abroad, or from public or private research centers.

L'archive ouverte pluridisciplinaire **HAL**, est destinée au dépôt et à la diffusion de documents scientifiques de niveau recherche, publiés ou non, émanant des établissements d'enseignement et de recherche français ou étrangers, des laboratoires publics ou privés.



For Peer Review Only

Validation of a wide spectrum microbiological tube test, the EXPLORER test, for the detection of antimicrobials in muscle from different animal species

Journal:	*Food Additives and Contaminants*
Manuscript ID:	TFAC-2008-433.R1
Manuscript Type:	Original Research Paper
Date Submitted by the Author:	27-Mar-2009
Complete List of Authors:	Gaudin, Valerie; AFSSA, LERMVD Hedou, Celine; AFSSA, LERMVD Verdon, Eric; afssa, lermvd
Methods/Techniques:	In-house validation, Inter-laboratory validation, Method validation, Screening - microbial screening
Additives/Contaminants:	Residues, Veterinary drug residues, Veterinary drug residues - antibiotics, Veterinary drug residues - antimicrobials
Food Types:	Animal products – meat, Meat

SCHOLARONE™
Manuscripts

Validation of a wide spectrum microbiological tube test, the EXPLORER® test, for the detection of antimicrobials in muscle from different animal species

V.GAUDIN*, C. HEDOU & E. VERDON

Community Reference Laboratory for Antimicrobial Residues, AFSSA Fougères – LERMVD, La Haute Marche – BP 90203, 35302 FOUGERES Cedex, France

*To whom correspondence should be addressed.

e-mail: v.gaudin@fougeres.afssa.fr

For Peer Review Only

Abstract

EXPLORER® is a simple and fast new kit for detection of inhibitory substances in raw meat. The test, a 96-well microtiter plate, is based on the inhibition of microbial growth *(Geobacillus stearothermophilus* spores). The Explorer® test was validated in accordance with the decision 2002/657/EC (EC 2002). The specificity and detection capabilities for 5 compounds from major antimicrobial families and robustness were studied. The specificity of the test was assessed with 4 different animal species and was found to be very satisfactory (false positive rates lower than 10 %). The detection capabilities for amoxicillin (10 µg kg^{-1}) and tylosin (100 µg kg^{-1}) were at the MRL level (50 and 100 µg kg^{-1} respectively) for both, for doxycycline (200 µg kg^{-1}) and sulfathiazole (200 µg kg^{-1}) at 2 times the MRL (100 µg kg^{-1} for each) and for cefalexin (500 µg kg^{-1}) at 2.5 times the MRL (200 µg kg^{-1}). Twenty-one samples were analysed in parallel with the Four Plate Test, the STAR protocol and the Explorer® test. One false positive result and 2 false negative results (samples containing oxytetracycline) were reported with the Explorer® test. In conclusion, the Explorer® test was shown to be robust and easily automated. Photometric reading allows informatic data storage and objective readings between technicians and days. The Explorer® test could be used as a wide screening test because it enables detection of most of the antimicrobial families (penicillins, cephalosporins, tetracyclines, sulphonamides and macrolides) in muscles from different animal species (porcine, bovine, ovine, poultry).

Keywords: *Validation, Explorer, inhibition test, antimicrobial screening assay, muscle samples, animal species, photometric reading*

For Peer Review Only

Introduction

The presence of antimicrobial residues in food of animal origin could be due to treatment of animal disease, or prophylaxis purposes. The risks for consumer health due to the presence of antimicrobial residues could be: toxicological effects, allergies (especially with beta-lactam residues), or antimicrobial resistance of pathogenic bacteria. Therefore, Maximum Residue Limits (MRLs) have been set for antimicrobial residues in the European Union (2377/90/EC) (EC 1990). There are a large number and the variety of antimicrobial families. Moreover, the antibiotics used in different countries are generally different. Therefore, the tests which are commercialised all over the world have to be able to detect a maximum number of these antibiotics having an MRL. More than sixty different antimicrobials have MRLs in animal tissues. So the detection of antimicrobial residues should be performed with wide screening tests, which are the first level of official control, at MRL level.

Different methods have already been developed and proposed for screening of antimicrobials in muscle. Different microbiological plate tests are available: Four Plate Test (FPT) (Bogaerts et al. 1980), a Five Plate Test developed at the Community Reference Laboratory (CRL) called the STAR protocol (Screening Test for Antibiotic Residues) (Gaudin et al. 2004), other plate tests: a one-plate method (Koenen-Dierick et al. 1995), a modified EC Four Plate Method to detect antimicrobial drugs (Okerman et al. 1998, Currie et al. 1998), six-plate tests for meat samples (Myllyniemi et al. 2001, Ferrini et al. 2006). The European Four Plate test is commonly used for detection of antibiotics in food. This method requires stabilisation of several bacteria strains and takes about 24 hours to produce results. Moreover, the detection capability of this assay is well above the MRL for sulphonamides.

More recently, some tube tests have been developed for the screening of antimicrobial residues in meat as proposed for milk analysis. Generally, these tube tests are based on the strain *Geobacillus stearothermophilus (formerly Bacillus stearothermophilus)*. The ampoule contains a pre-seeded medium, with a pH change indicator. The reading is based on the change of colour of the medium because of a redox indicator which produces a colour change in the test when

http://mc.manuscriptcentral.com/tfac Email: fac@tandf.co.uk

antibiotics are not present in the sample (yellow for negative samples, purple for positive samples). This kind of test is ready-to-use and easy-to-use, while plate tests are home-made methods and need to maintain bacterial strain. The first commercial tube test for muscle was the Premi®Test (DSM, Delft, The Netherlands). The incubation time of Premi®Test (2h30 to 2h45) is lower than for plate tests (15 to 24 hours). More recently, a new test has been developed by another company (Zeu-Inmunotec, Saragossa, Spain). This new test, called Explorer® test, is also based on the bacterial strain *Geobacillus stearothermophilus*. The principle of the test is the same as Premi®Test. However, Explorer® test uses a new microplate format (96 wells). The Explorer® test has been optimized for different meat species: pork, chicken, beef, lamb, etc. by the manufacturer.

In our lab, Premi®Test was fully validated for its use on muscles from different animal species (Gaudin et al. 2008)). The objective of the present study was to evaluate the performance characteristics of the Explorer® kit from Zeu-Inmunotec (Saragossa, Spain) for the detection of antibiotic residues in muscle from different animal species. To our knowledge, no studies of the performance of this test have been published at this time. This article will present the results of a preliminary validation study performed at the Community Reference Laboratory. The validation of screening tests for the detection of antimicrobial residues was conducted as in the decision EC/2002/657 (EC 2002). Detection capabilities (CCβ), specificity, false positive rate were calculated. Furthermore, a robustness study has been conducted on 4 parameters based on an experimental plan.

Materials and methods

Explorer® test

Explorer® test is a simple and fast kit for detection of inhibitory substances in raw meat, feed and eggs. The test, a 96-well microtiter plate, is based on the inhibition of microbial growth. Each well contains specific agar medium containing a redox indicator and spread with *Geobacillus stearothermophilus* spores.

For Peer Review Only

Extraction of muscle samples for Explorer® test. A piece of meat (2-3g) was cut and put into a clean glass or heat resisting plastic tube. The tube was closed and heated it in a water bath at 100℃ for 3 min. After the heating, some fluid was released from the sample. The meat sample was pressed with forceps to release as much fluid as possible. Finally the meat fluid was clarified by centrifugation (2000 g for 3 min).

Test procedure. 0.1 ml of meat fluid was added to each well with a micropipette. The wells were sealed with an adhesive sheet and the microplate was pre-incubated at 65ºC for 30 min in a block heater (ref. ZE/FX – FX incubator supplied by Zeu-Inmunotec, Saragossa, Spain) to allow the sample to diffuse through the well. Afterwards the wells were washed by filling the wells up with distilled water, using a squeeze bottle. The washing step was repeated 3 times. The plate was emptied by turning the plate upside down on top of an absorbent paper to remove the water in excess. The plate was sealed again with an adhesive sheet and incubated at 65ºC until the negative control sample has turned yellow (approximately 3h-3h30min).

Quality controls (QC)

It was strongly recommended by the manufacturer to use a negative control sample (antibiotic-free meat) to determine the optimal incubation time of each assay. It was also advisable to include a positive sample (meat sample with high concentration of antibiotic, i.e. Penicillin G 25 $\mu g kg^{-1}$ in juice extract). Penicillin G was supplied by Sigma-Aldrich (Saint-Quentin-Fallavier, France).

2 different negative controls were used:

- controls prepared with meat from the same animal species as the meat tested (prepared by ourselves),
- a control supplied by Zeu-Inmunotec (Saragossa, Spain) which was a mix of juices from different animal species (lyophilised form to be reconstituted).

Readings and interpretation of Explorer results. When the plate was incubated at 65ºC, spores germinate and cells grow modifying the redox potential of the medium The agar colour changed

For Peer Review Only

from blue to yellow if the sample did not contain antibiotics or antimicrobial residues at concentrations below the detection limits of the test. If samples contained antibiotics at concentrations above the detection limit of the kit, microorganisms will not grow neither colour changes will be observed.

The photometric reading of microplates is based on the reading at 2 wavelengths (590 and 650 nm) on a microplate reader. The results were interpreted as the difference between the values of the two readings.

An example of interpretation of quantitative results (Optical Density) is presented on Figure 1.

"[Insert Figure 1 about here]"

At each day of validation, one negative control of each species was analysed and also one positive control (penicillin G at 25 $\mu g kg^{-1}$). Then the results of each unknown sample from each species were compared to the corresponding negative control. The plate is ready to be read when the result for the negative control sample (difference of absorbance NA590nm - NA650 nm) between 0.15 and 0.25 OD (optical density units), where NA: Negative control absorbance. A sample is declared positive when: SA 590nm - SA 650 nm ≥ NA590nm - NA 650nm + 0.15, where : SA: Sample absorbance.

Validation

Specificity and false positive rate. 20 blank meat samples from different origins should be analysed in blind duplicate with Explorer® kit. The applicability of the test to meat from different animal species was studied simultaneously with specificity determination because the 20 meat samples were from different species: 5 porcine meat, 5 bovine meat, 5 poultry meat and 5 ovine meat.

Detection capabilities Five different antimicrobials were tested at the MRL and at the announced detection limit (manufacturer data): amoxicillin (penicillin family), cefalexin (cephalosporin family), doxycycline (tetracycline family), sulfathiazole (sulphonamide family) and tylosin (macrolide family).

For Peer Review Only

Antibiotic and sulphonamide standards were provided by Sigma Aldrich (Saint-Quentin Fallavier, France).

First the fluid from blank muscle samples was extracted by pressing the meat with a garlic press. Then the juice samples were spiked with antibiotic solutions. Therefore, the validated concentrations are the real concentrations in the juice samples. Twenty samples (5 porcine, 5 bovine, 5 ovine and 5 poultry) fortified at 2 levels with each antimicrobial were prepared "blind to the analyst" and analysed in duplicate on 4 different days (5 samples per day). Blank samples and spiked samples were aliquoted and stored frozen over 1 week maximum to prevent stability problems with antimicrobials. The blank samples could be analysed during a longer period because there is no problem of stability.

At the end of the validation, 5 spiked samples for each antibiotic at each concentration and 5 blank samples, from each species, have been analysed. In the case of substances with an established MRL, the detection capability is the concentration at which the method is able to detect a compound at the MRL with a statistical certainty of $1 - \beta$. The detection capability $CC\beta$ was calculated as the concentration where less than 5% of false compliant results remains. When one sample or less among 20 samples spiked at the MRL is detected negative, the detection capability was equal to or lower than the MRL.

Robustness study. Robustness studies use the deliberate introduction of minor reasonable variations by the laboratory and the observation of their consequences. Factors which may influence the measurement results have been selected: sample volume, pre-incubation time, pre-incubation temperature and incubation temperature. These factors should be modified in an order of magnitude that matches the deviations usually. The robustness study has been focused on one representative antimicrobial amoxicillin and on porcine muscle. 10 blank samples and 10 different samples, spiked at the detection capability of amoxicillin were blindly analysed, in duplicate, at different days.

http://mc.manuscriptcentral.com/tfac Email: fac@tandf.co.uk

For Peer Review Only

An experimental plan which combined the different factors was built to minimise the experiments. A full factorial design was chosen. Based design was 2-level factorial design for 3 factors (volume of samples, pre-incubation time and pre-incubation temperature), noted by letters A, B and C. For each of these factors, a low value ("-") and a high value ("+") (low and high compared to normal conditions) have been tested. Considering the 3 factors and 2 levels for each one ("-", "+"), the total number of experiments was 8 (8 different days). On each different day, 10 blank samples and 10 spiked samples have been analysed. Experimental runs were coded as '–' for low levels and '+' for high levels (Table I).

"[Insert Table I about here]"

A fourth factor (incubation temperature) has been studied by adding negligible interaction to the experimental design (Renard et al. 1992). Incubation temperature (letter D=ABC) was studied at levels defined by interaction between the 3 factors of the base design (ABC). "D" and "ABC" were alias structure. The design matrix is presented in Table II.

"[Insert Table II about here]"

Therefore, 4 factors have been studied (8 combinations of experiments = 8 runs = 8 days). The influence of these factors on false positive and false negative rates and on incubation time as well as the interactions between factors were evaluated. For each run (day), the response is indicated in Table III (false positive rate, false negative rate and on incubation time).

Comparison with other screening methods and confirmation of positive samples by LC/MS-MS (Incurred tissues). The Four Plate Test (FPT) is the French official method for the screening of antimicrobials in muscle (Bogaerts et al. 1980). The STAR protocol is a Five Plate Test which was developed and validated for the screening of antimicrobial residues in milk, by the Community Reference Laboratory for antimicrobial residues (AFSSA Fougères) (Gaudin et al. 2004). The STAR protocol is also applicable to the screening of antimicrobial residues in muscle and is in progress to be validated for muscle screening. The 2 plate tests are able to detect a wide range of antibiotics (beta-lactams, tetracyclines, macrolides, quinolones, aminoglycosides, sulfonamides). The detection limits of these tests are very different between families of antibiotics (beta-lactams and tetracyclines are the best detected) and between antibiotics inside each family. Moreover, the

For Peer Review Only

detection limits of the STAR protocol for most of the antibiotics are lower than detection limits of the FPT. It is particularly obvious with sulfonamides and quinolones. Twenty-one muscle samples from different animal species were collected from the field laboratories in France and were analysed in AFSSA Fougères in parallel with the Explorer® kit, the FPT and the STAR test. Finally, all the positive samples with at least the FPT or the STAR protocol were confirmed by a multi-residue LC/MS-MS method (Gaudin et al. 2008). The principle is based on two different extractions : one with trichloroacetic acid (TCA) allowing the detection of tetracyclines, aminoglycosides and quinolones, the second with acetonitrile (ACN) allowing the detection of penicillins, cephalosporins, macrolides and sulphonamides. LC/MS-MS is used with a Multi Reaction Monitoring Mode (MRM) and two MRM transitions are monitored for each compound. Fifty monitored compounds excepted some aminoglycosides were detected in muscle at a level below the MRL.

Results and discussion

Specificity and false positive rate

When the negative control was our internal control (one control per tested species), 19 of the 20 blank meat samples (5 bovine meat, 5 porcine meat, 5 poultry meat and 5 ovine meat) were found negative (1 bovine sample) (5% of false positive results). However, when the negative control was from Zeu-Inmunotec (Saragossa, Spain), 18 of the 20 blank meat samples (5 bovine meat, 5 porcine meat, 5 poultry meat and 5 ovine meat) were found negative (1 bovine and 1 porcine samples) (10% of false positive results). No influence of the animal species was observed on the specificity.

The false positive rate seemed to be higher (10 % instead of 5%) with the control from Zeu-Inmunotec (Saragossa, Spain). The consequence of a higher false positive rate at the screening step is that more confirmatory analyses have to be performed by physico-chemical methods. However, the most important performance characteristic for a screening method is that the detection capability should be as near as possible from the MRL and to have a false negative rate

For Peer Review Only

lower than 5%. Consumer safety is more important than the cost of analyses, even if the developer of screening methods have to be informed of the consequences of high false positive rates.

To increase the level of confidence in the determination of the false positive rate, more than 20 blank samples would have to be tested. This first determination gave us an estimation of the false positive rate. More experiments would have to be performed in the future to complete the validation.

Detection capabilities

The detection capabilities were calculated for the 5 tested antimicrobials and compared with those indicated by the manufacturer (Table III).

"[Insert Table III about here]"

The Maximum Residue Limit (MRLs) for amoxicillin, doxycycline, sulfathiazole and tylosin are respectively 50, 100, 100 and 100 µg kg^{-1}. The CCβ values of amoxicillin and tylosin were at the MRL level. The detection capabilities of doxycycline and sulfathiazole are at 2 times the MRL level and around 3 times the MRL level for cefalexin. The detection capabilities determined during this validation were similar to those claimed by the manufacturer. Two different negative controls have been tested and used to calculate the cut-off value to classify unknown samples as negative or positive. The first control fluid was prepared with meat from the same animal species as the meat tested (prepared by ourselves). The second control was supplied by Zeu-Inmunotec (Saragossa, Spain) and was a mix of juices from different animal species. The choice of the negative control from Zeu-Inmunotec (Saragossa, Spain) could be interesting regarding the following results. There were no influence of the negative control on the CCβ values of amoxicillin, tylosin, doxycycline and sulfathiazole. There was only a slight influence on the detection of cefalexin. The CCβ was a little lower when the negative control was that from Zeu-Inmunotec (Saragossa, Spain). Moreover, it would be easier to use a prepared negative control like it (lyophilised), instead of preparing a negative control, from each species, in the laboratory implementing the kit. Therefore, the use of

For Peer Review Only

this negative control could be recommended for practical, technical and economical reasons, even if the false positive rate seems to be higher (10 % instead of 5 %).

The detection capabilities were calculated after the analyses of muscles from 4 different species. No influence of the animal species was observed on the CCβ values. The same conclusion was reported with the Premi®Test by Cantwell et al. (2006).

Robustness study

The influence of 4 factors (volume of samples, pre-incubation time, pre-incubation temperature and incubation temperature) on false positive and false negative rates and on incubation time as well as the interactions between factors were evaluated. For each run (day), the response is indicated in Table III (false positive rate, false negative rate and on incubation time). These values were added together. Than the result was divided by the number of runs (8 in this case). A quantitative value of the effect of each factor in relation to average of runs (column noted I was then obtained (Table IV).

"[Insert Table IV about here]"

False positive rate. The false positive rate did not change with sample volume (A), pre-incubation time (B) and pre-incubation temperature (C). A negative effect of interaction D=ABC was observed on false positive rate, that means that false positive rate decreased when factor D (incubation temperature) increased. On the contrary, a positive effect of interaction AB+CD and AC+BD was observed. The false rate positive rate increased when these factors interacted.

False negative rate. The false negative rate decreased with sample volume (A) and pre-incubation time (B) and with the interaction BC+AD, that means that the false negative rate decreased when sample volume and pre-incubation time increased. This conclusion was not surprising because it is well known that for these kind of tests, the detection capability of the test is better if the sample volume is higher and pre-incubation time longer. A negative effect on false negative rate was observed with interaction BC+AD. Therefore, the interaction of these factors reduced the false negative rate. However, a positive effect of D=ABC (incubation temperature) and

For Peer Review Only

interactions AB+CD and AC+BD. Therefore, the false negative rate increased when incubation temperature increased. The most important effect was observed on run 5 where volume of samples and pre-incubation time were lowered and pre-incubation temperature and incubation temperature were increased. The false negative rate was equal to 27.3 %. Pre-incubation temperature (C) had no effect on the false negative rate. In the protocol of Explorer® test, there is no recommended standard deviation on pre-incubation and incubation temperature at 65°C. This study proved that pre-incubation temperature could vary from 62 to 68°C without influence on the detection capability of amoxicillin. However, the false negative rate increased when incubation temperature increased.

Incubation time. Factor B (pre-incubation time) had the most important effect on the incubation time. When pre-incubation time decreased, incubation time increased and conversely. It is well known that for these kind of tests, the detection capability of this kind of test decreased when incubation time is too long. However, as the end-point of the test is based on the optical density of the negative control and not on a finite time, this consideration does not affect the ruggedness of the assay.

As a conclusion, individual factors (except factor D) had few or not at all impact on the detection capability or specificity of the test. Factor D (incubation temperature) increased the false negative rate significantly. When incubation temperature increased, false negative rate increased and false positive rate decreased. Factor D The incubation temperature was the most critical factor. Finally, the interactions of factors AB+CD and AC+BD had a bad effect on the performance of the test because false positive and false negative rates increased.

The requirements regarding a screening test for veterinary drugs are a false negative rate equal or lower than 5% and a false positive rate as low as possible. Therefore, the Explorer® test was found to be rugged with respect to animal species, sample volume, pre-incubation time, pre-incubation temperature and incubation temperature.

For Peer Review Only

Comparison with other screening methods and confirmation of positive samples by LC/MS-MS (Incurred tissues). Twenty-one incurred muscle samples from the field laboratories were analysed with 3 microbiological methods. Different animal species were analysed: 13 bovine, 6 porcine, 1 turkey, 1 unknown. These samples were taken randomly in the pool of samples arriving in our laboratory. The results of all analyses are presented in Table V. At least 7 samples contained very high concentrations of antimicrobials. Therefore, these samples were easily detected by the 3 microbiological tests (Explorer® kit, Four Plate test and STAR test).

"[Insert Table V about here]"

The samples which were detected positive with one screening test, but which finally did not contain antimicrobial residues or residues at concentrations lower than MRL (after the LC/MS-MS confirmation) were considered as false positive results. Seven false positive results were reported with the STAR protocol and 4 with the FPT. For 3 of them (2 porcine and 1 bovine samples), the explanation was that these 2 methods were able to detect tetracyclines, even at concentrations lower than MRL (too sensitive).

Concerning the 4 other false positive results obtained with the STAR protocol and the one remaining false positive result of the FPT (1 bovine sample), either it was real false positive results (presence of natural inhibitors) or the antimicrobial residue was one of those which was not detected by the multi-residue LC/MS-MS method. The good example is sample codified v-1510 which was positive with both FPT and STAR protocol, but confirmed negative by LC/MS-MS. Only 1 false positive result was obtained with the Explorer® test (bovine sample). However, because this sample was negative with the STAR protocol and the FPT (official method), it was not confirmed by LC/MS-MS. Therefore, it was impossible to be sure that it was a real false positive result.

Only the samples really containing antimicrobials at concentrations upper than MRL and not detected by the screening test were considered as false negative results. The STAR protocol did not give any false negative results (0%). The Four Plate Test (FPT) gave 1 false negative result (4.7%) and the Explorer® test 2 false negative results (9.5%). The FPT failed to detect amoxicillin

For Peer Review Only

in a bovine sample, at a concentration higher than the MRL. The Explorer® test did not detect oxytetracycline in 2 different samples: a turkey sample at 140 µg kg^{-1} and a bovine sample at 315 µg kg^{-1}. This was not surprising because with most of the microbiological tests, tetracyclines are not generally detected at the MRL level (100 µg kg^{-1}), especially when only one bacterial strain was used for detection (i.e. Premi®Test) (Gaudin et al. 2008), but a little higher. Furthermore, when the incubation increased, the detection capability decreased rapidly, especially for tetracyclines. Okerman et al. (2004) also reported a poor detection capability of Premi®Test towards tetracyclines in muscle tissue. At the MRL level, no positive results over 3 tests were reported for the 4 tetracyclines (tetracycline, oxytetracycline, chlortetracycline and doxycycline). Moreover, incurred chicken tissue with doxycycline at 108 µg kg^{-1} was found negative also. Premi®Test was sensitive enough for sulphonamides and some macrolides. The Explorer® test was also found sensitive enough for 2 antibiotics of these families (sulfathiazol and tylosin).

- Two points should be underlined concerning the filed samples: firstly, some antibiotics were present at very high concentrations, much higher than MRLs.
- Secondly, 9 samples out of 21 contained several antibiotics from the same family and/or from different families.

The high concentrations contained in real samples sometimes could be due to the fact that the sample has been taken at injection point. Moreover, samples with the concentrations nearest from the respective MRL of antimicrobials should be more interesting to conclude on the performance of the tests. However, field samples were randomly taken and before confirmatory analyses, we had no idea of the antimicrobial concentration. For this reason, a parallel validation with spiked juice samples at the target concentration (MRL) was really necessary to determine detection capabilities of the Explorer® test. To our knowledge, there were no scientific publications at this time presenting results obtained with the Explorer® test. However, there were some validation studies performed on the Premi®Test. In the classical protocol of Premi®Test, there is no solvent extraction of muscle samples, only fluid extraction with a garlic press. Stead et al. (2004) proposed an extraction protocol (solvent extraction) before using the Premi®Test. The validation study compared the detection limits of the 2 different protocols. The detection limits were generally lower

For Peer Review Only

with the solvent extraction than with the fluid extraction. The detection limits with a fluid extraction only for amoxicillin, doxycycline, sulfathiazole and tylosin were respectively > 5, 50, 25 and <25 µg kg^{-1}. Here, the detection capabilities of the Explorer® Test for amoxicillin, doxycycline, sulfathiazole and tylosin were respectively 10, 200, 200 and 100 µg kg^{-1}. Therefore, the detection capability of Premi®Test even with fluid extraction only, seemed to be better than the detection capability of the Explorer® test. However, the detection limits and the CCβ values were not determined in the same way. Stead et al. (2004) determined the detection limits by analysing n=4 spiked samples for each analyte/matrix combination. The detection capability was determined for the Explorer® test after the analyses of 20 samples and the CCβ was calculated as the antibiotic concentration given less than 5 % of false negative results. Therefore, the values were not directly comparable. Furthermore, in our lab, the Premi®Test was validated for the detection of antimicrobial residues in muscle (Gaudin et al. 2008)). The results were similar to those obtained with the Explorer® test during our validation study for the 2 common tested antibiotics, amoxicillin (CCβ ≤ 50 µg kg^{-1}) and tylosin (CCβ = 100µg kg^{-1}).

The photometric reading of the Explorer® test was very interesting. It is well known that visual evaluation of this kind of test is subjective. As it was observed with the Premi®Test (Stead et al. 2005), the use of a scanner technique removes the subjectivity of the analyst involved in visually determining the end-point colour. The same conclusion could be underlined with the Explorer® test and the photometric reading. The determination of a cut-off value from the quantitative value of the negative control in the Explorer® protocol allows to classify negative and positive results from an objective parameter.

Conclusion

The Explorer® test (Zeu Inmunotech, (Saragossa, Spain) is applicable to the detection of different antimicrobial families (penicillins, cephalosporins, tetracyclines, macrolides and sulfonamides) in the muscles from different animal species (bovine, porcine, ovine and poultry). The detection capabilities of the 5 tested antimicrobials were at the MRL level or lower (amoxicillin, tylosin) and

For Peer Review Only

sometimes a little higher than the respective MRLs (sulfathiazole, doxycycline, cefalexin) (2 to 3 times the MRL). The applicability of Explorer® test to muscles from different animal species was proved when a negative control from each species is tested for each assay. The sample preparation is quick and simple. This test could be easily automated because of the microplate format. High throughput of samples could be analysed. Furthermore, the advantages of photometric reading were the objectivity of results avoiding variations due to visual reading made by different technicians or performed over different days. Finally, spectrophotometric reading allowed informatic registration, ensuring traceability of assays.

The results of Explorer® test for the analysis of incurred field samples were very satisfactory. This demonstrates that this technique seems to be fit-for-purpose as a qualitative screening assay. These results were very promising. However, some families of antimicrobials have not been tested yet (e.g. aminoglycosides, quinolones). These families are generally not well detected by microbiological tests, especially when all only bacterial strain is used. The next step should be to validate the test for other families of antibiotics and a higher number of antimicrobials from the main families.

Acknowledgements

Thanks to Zeu-Inmunotec (Saragossa, Spain) for providing us with different batches of EXPLORER® test.

References

Bogaerts R, Wolf F. 1980. A standardized method for the detection of residues of antibacterial substances in fresh meat. Die Fleischwirtschaft 60:672-674.

Cantwell H, O'Keeffe M. 2006. Evaluation of the Premi Test and comparison with the One-Plate Test for the detection of antimicrobials in kidney. Food Additives and Contaminants 23(2):120-125.

For Peer Review Only

Currie D, Lynas L, Kennedy DG, McCaughey WJ. 1998. Evaluation of a modified EC Four Plate Method to detect antimicrobial drugs. Food Additives and Contaminants 15:651-660.

EC 1990. Council Regulation (EEC) N° 2377/90 of 26 June 1990: laying down a Community procedure for the establishment of maximum residue limits of veterinary medicinal products in foodstuffs of animal origin. Official Journal of European Communities L224:1-8.

EC 2002. Commission Decision 2002/657/EC of 12 August 2002: implementing Council Directive 96/23/EC concerning the performance of analytical methods and the interpretation of results. Official Journal of European Communities L221:8-36.

Ferrini AM, Mannoni V, Aureli P. 2006. Combined Plate Microbial Assay (CPMA): A 6-plate-method for simultaneous first and second level screening of antibacterial residues in meat. Food Additives and Contaminants 23:16-24.

Gaudin V, Maris P, Fuselier R, Ribouchon JL, Cadieu N, Rault A. 2004. Validation of a microbiological method: the STAR protocol, a five-plate test, for the screening of antibiotic residues in milk. Food Additives and Contaminants 21:422-433.

Gaudin V, Juhel-Gaugain M, Moretain JP, Sanders P. 2008. AFNOR validation of Premi®Test, a microbiological-based screening tube-test for the detection of antimicrobial residues in animal muscle tissue. Food Additives and Contaminants 25(12): 1451–1464.

Koenen-Dierick K, Okerman L, De Zutter L, Degroodt JM, Van Hoof J, Srebnik S. 1995. A one-plate microbiological screening test for antibiotic residue testing in kidney tissue and meat : an alternative to the EEC four-plate method?. Food Additives and Contaminants 12:77-82.

Myllyniemi AL, Nuotio L, Lindfors E, Rannikko R, Niemi A, Backmann C. 2001. A microbiological six-plate method for the identification of certain antibiotic groups in incurred kidney and muscle samples. Analyst 126:641-646.

Okerman L, Van Hoof J, Debeuckelaere W. 1998. Evaluation of the European four-plate test as a tool for screening antibiotic residues in meat samples from retail outlets. Journal of AOAC International 81:51-56.

Okerman L, Croubels S, Cherlet M, De Wasch K, De Backer P, Van Hoof J. 2004. Evaluation and establishing the performance of different screening tests for tetracycline residues in animal tissues. Food Additives and Contaminants 21:145-153.

For Peer Review Only

Renard L, Moulin G, Sanders P. 1992. Using experimental design to optimize a diffusion assay. Journal of AOAC International 75 (6):1045-1048.

Stead S, Sharman M, Tarbin JA, Gibson E, Richmond S, Stark J, Geijp E. 2004. Meeting maximum residue limits: an improved screening technique for the rapid detection of antimicrobial residues in animal food products. Food Additives and Contaminants 21:216–221.

Stead S, Richmond S, Sharman M, Stark J, Geijp E. 2005. A new approach for detection of antimicrobial drugs in food — Premi®Test coupled to scanner technology. Analytica Chimica Acta 529:83–88.

For Peer Review Only

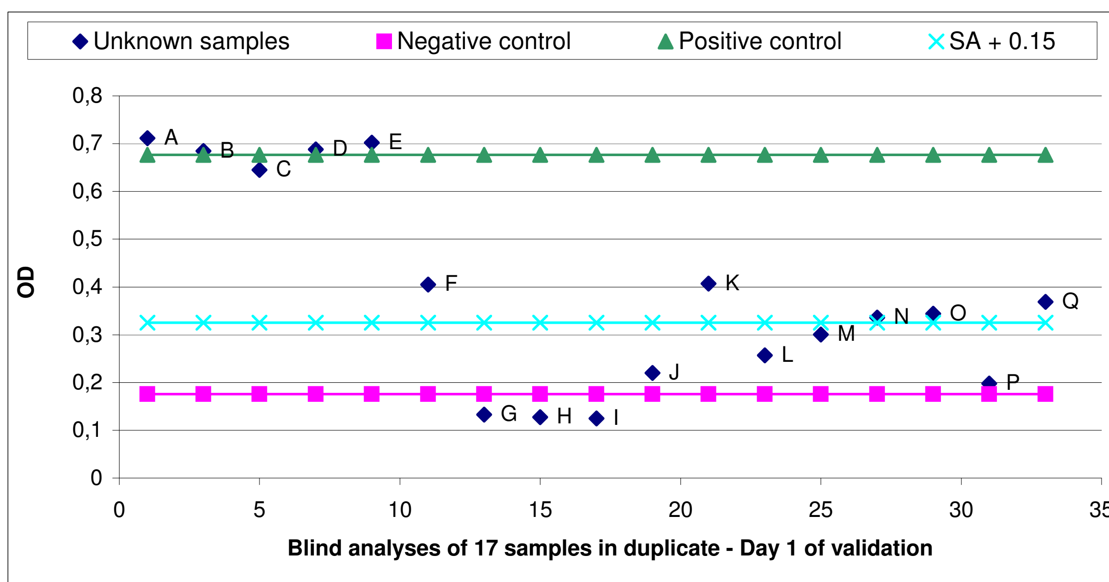
Figure 1. Example of raw data for day 1 of validation: Interpretation of the results.

At each day of validation, one negative control of each species was analysed and also one positive control (penicillin G at 25 µgkg-1). Then the results of each unknown sample from each species were compared to the corresponding negative control. The plate was ready to be read when the result for the negative control sample (difference of absorbance NA590nm - NA650 nm) was between 0.15 and 0.25 OD (optical density units) where NA: Negative control absorbance. A sample is declared positive when: SA 590nm - SA 650 nm ≥ NA590nm - NA 650nm + 0.15 where : SA: Sample absorbance.

For Peer Review Only

1
2
3
4
5
6
7
8
9
10
11
12
13
14
15
16
17
18
19
20
21
22
23
24
25
26
27
28
29
30
31
32
33
34
35
36
37
38
39
40
41
42
43
44
45
46
47
48
49
50
51
52
53
54
55
56
57
58
59
60

Figure 1. .



Peer Review Only

1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17 18 19 20 21 22 23 24 25 26 27 28 29 30 31 32 33 34 35 36 37 38 39 40 41 42 43 44 45 46 47 48 49 50 51 52 53 54 55 56 57 58 59 60

1 *Table I. 2-level experimental design with 4 factors: levels of each factor.*

4 tested factors:	**Level "-"**	**Level "+"**	**Normal conditions**
A- Volume of samples	80 µl	120 µl	100 µl
B- Pre-incubation time	20 min	40 min	30 min
C- Pre-incubation temperature	62°C	68°C	65°C
D- Incubation temperature	62°C	68°C	65°C

2
3
4
5

For Peer Review Only

1
2 6 *Table II. Robustness study: Design matrix and experimental design calculation.*
3
4
5

	Levels										
Run	I	A[a]	B[b]	C[c]	D[d] = ABC	AB+CD	AC+BD	BC+AD	False positive rate	False negative rate	Incubation time
1	+	-	-	-	-	+	+	+	10	0	3h30
2	+	+	-	-	+	-	-	+	0	0	3h45
3	+	-	+	-	+	-	+	-	0	0	3h25
4	+	+	+	-	-	+	-	-	10	0	3h10
5	+	-	-	+	+	+	-	-	0	27,3	3h35
6	+	+	-	+	-	-	+	-	0	0	3h50
7	+	-	+	+	-	-	-	+	0	0	3h05
8	+	+	+	+	+	+	+	+	0	0	2h55

7 [a] *Volume of samples;* [b] *Pre-incubation time;* [c] *Pre-incubation temperature;* [d] *Incubation temperature*

8

9

10 11 12 13 14 15 16 17 18 19 20 21 22 23 24 25 26 27 28 29 30 31 32 33 34 35 36 37 38 39 40 41 42 43 44 45 46 47 48 49 50 51 52 53 54 55 56 57 58 59 60

For Peer Review Only

Table III. Detection capabilities of the 5 tested antimicrobials (in µg kg-1).

	Antimicrobial family	PENICILLINS		CEPHALOSPORINS		TETRACYCLINES		SULPHONAMIDES		MACROLIDES	
	Antimicrobial compound	Amoxicillin		Cefalexin		Doxycycline		Sulfathiazole		Tylosin	
	MRL muscle (µg kg-1)	**50**		**200**		**100**		**100**		**100**	
	Announced detection limit (µg kg-1)*	8		400-600		150-200		100-200		80-160	
	2 tested concentrations (µgkg-1)**	10	50	200	500	100	200	100	200	100	150
Internal negative control	Percentage of positive results (%)	100	100	30	85	40	100	65	100	100	100
	CCβ (µg kg-1) Explorer	**10**		**>500**		**200**		**200**		**100**	
Negative control Zeu-Inmunotec	Percentage of positive results (%)	100	100	30	100	45	100	75	100	100	100
	CCβ (µg kg-1) Explorer	**10**		**500**		**200**		**200**		**100**	

** by the manufacturer*

*** for the determination of detection capabilities*

For Peer Review Only

1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17 18 19 20 21 22 23 24 25 26 27 28 29 30 31 32 33 34 35 36 37 38 39 40 41 42 43 44 45 46 47 48 49 50 51 52 53 54 55 56 57 58 59 60

14 *Table IV. Mean of runs: effects of factors and interactions.*

	Factor				Interaction			Mean
Response	A	B	C	D=ABC	AB+CD	AC+BD	BC+AD	I
False positive rate	0.00	0.00	0.00	-2.50 [e]	2.50 [e]	2.50 [e]	0.00	2.50
False negative rate	-3.41 [e]	-3.41 [e]	0.27	3.41 [e]	3.41 [e]	3.41 [e]	-3.41 [e]	3.41
Incubation time	0.01	-0.26	0.07	0.01	-0.11	-0.11	-0.09	3.41

15 *[a] Volume of samples; [b] Pre-incubation time; [c] Pre-incubation temperature; [d] Incubation temperature*

16 *[e] Significant effect*

17

For Peer Review Only

Table V. Results of the comparative study between EXPLORER®, STAR protocol and the multi-residue LC/MS-MS method.

					LC/MS-MS*			
Internal sample code	Animal specie	Explorer®	FPT	STAR protocol	Identification	Quantification (µg kg^{-1})	MRL (µg kg^{-1})	< or > MRL
v-1487	Bovine	+	+	+	Oxytetracycline	~287	100	>
v-1489	Bovine	+	+	+	Penicillin G, dihydrostreptomycin, Oxytetracycline	~ 27850, ~ 28050, ~293	50 500 100	>
v-1490	Bovine	+	+	+	Dihydrostreptomycin	~54950	500	>
v-1492	Bovine	+	+	+	Oxytetracycline, Tetracycline	~33700, ~1470	100 100	>
v-1496	Unknown	+	+	+	Penicillin G, dihydrostreptomycin, doxycycline, chlortetracycline	~125, ~220, ~7.53, ~2.83	50 500 100 100	>
v-1501	Bovine	+	+	+	Sulfadoxine, Ciprofloxacin, Enrofloxacin, oxolinic acid	~ 1.46, ~1600, ~9500, ~7.4	100 100 100 100	>
v-1504	Bovine	+	-	+	Amoxicillin	~ 482	50	>
v-1499	Porcine	+	+	+	Oxytetracycline	~ 3140	100	>
v-1500	Bovine	+	+	+	Sulfamethoxypyridazine	~9175	100	>
v-1506	Turkey	-	+	+	Oxytetracycline	~ 140	100	>
v-1509	Bovine	-	+	+	Oxytetracycline	~ 315	100	>
v-1512	Bovine	+	+	+	Oxytetracycline, spiramycin, neospiramycin	~ 860.5, ~ 2410, ~ 263	100 200 200	>
v-1515	Bovine	+	+	+	Doxycycline, Oxytetracycline	~ 454, ~ 44	100 100	>
v-1495	Bovine	-	+	+	Tetracycline	~ 18.5	100	<
v-1497	Porcine	-	+	+	Tetracycline, chlortetracycline	~1.47, ~21	100 100	<
v-1498	Porcine	-	+	+	Sulfadiazine, chlortetracycline	~16, ~79	100 100	<
v-1513	Porcine	-	-	D	Traces of flumequine and spiramycin	NQ	200 250	<
v-1514	Porcine	-	-	+	Traces of flumequine	NQ	200	<
v-1508	Porcine	-	-	+	Negative	/	/	/
v-1510	Bovine	-	+	+	Negative	/	/	/
v-1488	Bovine	+	-	-	Not analysed	/	/	/

**Only positive samples with at least one of the screening method have been confirmed.*

FPT: Four Plate Test

D: Doubtful

NQ: Not Quantified

For Peer Review Only