

**AFNOR validation of Premi Test, a  
microbiological-based screening tube-test for the  
detection of antimicrobial residues in animal muscle  
tissue.**

Valerie Gaudin, Murielle Juhel-Gaugain, Jean-Pierre Morétain, Pascal Sanders

► **To cite this version:**

Valerie Gaudin, Murielle Juhel-Gaugain, Jean-Pierre Morétain, Pascal Sanders. AFNOR validation of Premi Test, a microbiological-based screening tube-test for the detection of antimicrobial residues in animal muscle tissue.. Food Addit Contam Part A Chem Anal Control Expo Risk Assess, 2008, 25 (12), pp.1451-1464. <10.1080/02652030802429088>. <hal-00606192>

**HAL Id: hal-00606192**

**<https://hal-anses.archives-ouvertes.fr/hal-00606192>**

Submitted on 5 Jul 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.



**AFNOR validation of PremiTest, a microbiological-based screening tube test for the detection of antimicrobial residues, in muscles from different animal origins**

Journal:	<i>Food Additives and Contaminants</i>
Manuscript ID:	TFAC-2008-016.R1
Manuscript Type:	Original Research Paper
Date Submitted by the Author:	30-Jul-2008
Complete List of Authors:	Gaudin, Valerie; AFSSA, LERMVD, CRL for antimicrobial residues in food Gaugain-Juhel, Murielle; AFSSA, LERMVD; AFSSA, LERMVD, CRL for antimicrobial residues in food Moretain, Jean Pierre; Agence Francaise de Securite Sanitaire des Aliments-CRL for antimicrobial residues in food, Laboratory for the research and study of veterinary medicinal products and disinfectants Sanders, Pascal; AFSSA, LERMVD
Methods/Techniques:	In-house validation, Inter-laboratory validation, Screening - microbial screening, Screening assays
Additives/Contaminants:	Veterinary drug residues - antibiotics, Veterinary drug residues - antimicrobials
Food Types:	Animal products – meat, Meat

SCHOLARONE™  
 Manuscripts

1 **AFNOR validation of Premi®Test, a microbiological-based screening tube test**  
2 **for the detection of antimicrobial residues, in muscles from different animal**  
3 **origins**

4  
5  
6 Valérie Gaudin\*, Murielle Juhel-Gaugain, Jean-Pierre Morétain, Pascal Sanders

7  
8 Community Reference Laboratory

9 AFSSA Fougères – LERMVD

10 La Haute Marche – BP 90203

11 35302 FOUGERES Cedex

12 France

13  
14  
15  
16 \*Address for correspondence : E-mail: [v.gaudin@fougeres.afssa.fr](mailto:v.gaudin@fougeres.afssa.fr)

17

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

## 18 Abstract

19 Premi®Test contains viable spores of a strain of *Bacillus stearothermophilus* which is  
20 sensitive to antimicrobial residues such as beta-lactams, tetracyclines, macrolides, and  
21 sulphonamides etc. The growth of the strain is inhibited by the presence of antimicrobial  
22 residues in muscle samples. Premi®Test was validated according to AFNOR rules (French  
23 Association for Normalisation). The AFNOR validation was based on the comparison of a  
24 reference method [French Official method (Four Plate Test) and the STAR protocol (5 plate  
25 test)] with the alternative method (Premi®Test). A preliminary study was conducted in an  
26 expert laboratory (Community Reference Laboratory CRL) on both spiked and incurred  
27 samples (field samples). Several method performance criteria (sensitivity, specificity, relative  
28 accuracy) were estimated and are discussed, in addition to detection capabilities. Adequate  
29 agreement was found between alternative methods and the reference method. However,  
30 Premi®Test was more sensitive to beta-lactams and sulphonamides than the FPT.  
31 Subsequently, a collaborative study with 11 laboratories was organised by the CRL. Blank  
32 and spiked meat juice samples were sent to participants. The expert laboratory (CRL)  
33 statistically analysed the results. It was concluded that Premi® Test could be used for the  
34 routine determination of antimicrobial residues in muscle of different animal origin with  
35 acceptable analytical performance. The detection capabilities of Premi®Test for beta-lactams  
36 (amoxicillin, ceftiofur), one macrolide (tylosin) and tetracycline were at the level of the  
37 respective Maximum Residue Limits (MRL) in muscle samples or even lower.

38  
39 **Keywords:** *Validation, Premi®test, screening, antimicrobial residues, muscle, collaborative*  
40 *study, routine analysis*

## 41 Introduction

42 Animal treatment can lead to the presence of residues in food of animal origin. The presence  
43 of antimicrobial residues could lead to human safety problems such as allergies or toxicity  
44 when foodstuffs containing residues enter the food chain. Different families of antimicrobial  
45 residues are concerned: beta-lactams, sulfonamides, macrolides, tetracyclines, quinolones,  
46 etc. For these reasons, Maximum Residue Limits (MRL) were set for many antimicrobial  
47 residues to protect consumer safety (EEC Directive 2377/90 and amendments). Different  
48 methods were developed for the detection of antimicrobial residues in food of animal origin.  
49 These screening methods are usually microbiological methods which are based on the  
50 inhibition of bacterial growth by antimicrobial residues. Microbiological plate tests generally  
51 give results in 18 to 24 hours. However, these home-made methods require skilled  
52 technicians, with a specific training. Microbiological based tests are interesting at the  
53 screening step because they are able to detect a wide range of antimicrobial residues  
54 (several families of antimicrobials are detectable with one single method). The levels of  
55 detection of these methods are generally satisfactory with respect to European regulations  
56 (EEC, 1990) for beta-lactams, tetracyclines and macrolides (detection capabilities lower or  
57 equal to the MRL). The best detected class of compound is generally the beta-lactam family.  
58 The detection capabilities of sulphonamides are very variable depending on the  
59 sulphonamide structure. The least detected antibiotics are generally some sulphonamides,  
60 aminoglycosides and quinolones by microbiological based tests. None of the existing  
61 microbiological screening methods are able to detect all the MRL substances in animal  
62 matrices. Therefore, some countries implemented other kind of methods in parallel to look for  
63 these antimicrobials specifically. In France, the official method for the control of muscle  
64 samples is the Four Plate Test (FPT) (Bogaerts and Wolf 1980). In the past, some  
65 commercial tube tests already exist but only for the analysis of milk, like Delvotest® and  
66 since a few years COPAN® test. These tests are based on the detection of growth by  
67 production of acid, visible by a colour change of the test medium (pH indicator). More  
68 recently, the Premi®Test, a commercial growth inhibitor test, was developed for the detection

69 of antimicrobial residues in muscle by DSM (DSM Food Specialities R&D, Delft, the  
70 Netherlands). This test is based on the inhibition of the strain *Bacillus stearothermophilus*.  
71 Premi®Test allows to detect antimicrobial residues in muscle in less than 4 hours (Fabre  
72 2003; Stead. 2004; Fabre *et al.* 2004)).

73  
74 Over the last few years, several evaluations of the Premi®Test have already been published,  
75 based on spiked meat juice samples (Reybroeck 2000a) or incurred poultry muscles  
76 (Reybroeck 2000b). Premi®Test has generally been compared to a reference method. Some  
77 studies were focused on a family of antimicrobials like beta-lactams, using spiked meat juice  
78 samples and incurred poultry muscle samples (Popelka *et al.* 2005) or tetracyclines  
79 (Okerman *et al.* 2004). The detection limits of different antimicrobials, calculated in spiked  
80 juice samples, were very near in the different studies. The global conclusions of the different  
81 studies were that Premi®Test is not suited for the detection of tetracyclines at MRL level in  
82 spiked and incurred samples. Moreover, Premi®Test was very sensitive for the screening of  
83 beta-lactams, more than usual microbiological plate tests. Premi®Test is suited for the  
84 detection of beta-lactams and sulphonamides, at MRL level or even below MRL.

85  
86 The AFNOR validation is based on the comparison between a reference method and an  
87 alternative method. In the present study, the Premi®Test, the alternative method, was  
88 compared with 2 other microbiological methods: the French Official method (Four Plate Test)  
89 which was the reference method and the STAR protocol (the CRL protocol) (Gaudin *et al.*  
90 2004). Afterwards, an interlaboratory study was organised, where only the Premi®Test has  
91 been used to analyse spiked meat juice samples. This paper presents the results of  
92 preliminary and collaborative studies.

## 94 **Materials and methods**

95 *Presentation of the methods*

96 *Principle of the kit.* The Premi®Test allows to detect antimicrobial residues in fresh meat,  
97 kidneys, fish and eggs (Arts et al. 2000). The Premi®Test is based on the growth inhibition of  
98 *Bacillus stearothermophilus*. Standardized spores are included in a medium, with selected  
99 nutrients. The meat juice was put on ready-to-use tubes. After 20 min of pre-diffusion at room  
100 temperature, the meat juice was removed by three washing steps. Finally, the ampoule was  
101 incubated during approximately 3 hours at 64°C. The reading of the "yes/no" result was  
102 based on a colour comparison. Without antimicrobials, the spores germinated and  
103 developed, involving the acidification of the medium and a change of colour (yellow).  
104 Conversely, in the presence of antimicrobials, the bacterial growth was inhibited. A purple  
105 colour indicated the presence of antibiotics, at or above the detection limit of the test.  
106 Doubtful and positive samples were confirmed by the multi-residue LC/MS-MS method  
107 described below. This broad spectrum test makes it possible to detect a great number of  
108 antimicrobials usually used, in less than 4 hours, on muscle juice samples (extracted by  
109 pressing a piece of meat).

111 *Principle of the Four Plate Test (FPT).* The Four Plate Test is the French Official method for  
112 the control of muscle samples (Bogaerts and Wolf 1980). A microorganism sensitive to  
113 antibacterial substances is inoculated into an agar medium in a Petri dish. The following test  
114 organisms are used: *Bacillus subtilis* BGA (reference 10649, Merck) (in test agar pH 6,  
115 Merck), *Bacillus subtilis* BGA (reference 10649, Merck) (in test agar pH 8, Merck), *Bacillus*  
116 *subtilis* BGA (reference 10649, Merck) (in agar ASS pH 7.4, Merck) and *Kocuria varians*  
117 (ATCC 9341, Pasteur Institute) (in test agar pH 8, Merck). Slices of frozen muscle were  
118 placed on the surface of the inoculated medium, and then incubated at the optimal  
119 temperature for growth of the test organism. After diffusion, the presence of antibacterial  
120 substance should produce an inhibition zone around the sample by inhibiting the growth of  
121 the test organism.

122

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

123 *Principle of the STAR protocol and Bacillus cereus.* The STAR protocol is the CRL method  
124 (Gaudin et al. 2004) which was developed to improve the performance of the Four Plate  
125 Test. The detection principle is the same as Four Plate Test. The following test organisms  
126 are used: *Bacillus subtilis* BGA (reference 10649, Merck) (Antibiotic medium II at pH 8.0,  
127 Difco), *Kocuria varians* ex. *Micrococcus luteus* (ATCC 9341, Pasteur Institute) (in test agar at  
128 pH 8, Merck), *Bacillus cereus* Bc6 (ATCC 11778, Pasteur Institute) (in test agar at pH 6,  
129 Merck), *Escherichia coli* (ATCC 11303, Pasteur Institute) (in test agar at pH 8, Merck),  
130 *Bacillus stearothermophilus* (ATCC 10149, Merck) (in DST (Diagnostic Sensitive Test)  
131 medium, Oxoid). The plate *Bacillus cereus* which was used by the field laboratories at the 3<sup>rd</sup>  
132 step of the preliminary study is the plate Bc6 of the STAR protocol, which is selective for the  
133 detection of the tetracyclines' family. Slices of muscle samples of 2 mm in thickness are cut  
134 in frozen muscle and put on the plates.

135  
136 *LC/MS-MS method.* A multi-residue LC/MS-MS method was developed for the screening of  
137 antimicrobials in meat. The principle is based on two different extractions : one with  
138 trichloroacetic acid (TCA) allowing the detection of tetracyclines, aminoglycosides and  
139 quinolones, the second with acetonitrile (ACN) allowing the detection of penicillins,  
140 cephalosporins, macrolides and sulfonamides. TCA extracts are directly injected after  
141 ultracentrifugation and filtration. ACN extracts are evaporated and the residue is then  
142 dissolved in 0.6 ml of ammonium acetate before filtration and injection. Two different  
143 gradients with pentafluoropropionic acid and ACN are used for the LC analyses. LC/MS-MS  
144 is used with a Multi Reaction Monitoring Mode (MRM) and two MRM transitions are  
145 monitored for each compound. The identification of the detected compounds is based on the  
146 retention time and the presence of the two specific transitions. The quantitative determination  
147 was carried out by using calibration curves obtained with spiked samples at 0.5 MRL, 1 MRL  
148 and 1.5 MRL levels. Internal standard were used for the quantification. All the 50 monitored  
149 compounds except some aminoglycosides were detected at a level below the MRL.

150



151 *Principle of AFNOR (French Association for Normalisation) validation*

152 An alternative method should be compared with a reference method. A reference method  
153 could be a standardized method when it exists, an official method, or a widely known and  
154 used method, taken in reference. An alternative method is a commercial test allowing to  
155 analyze, for a category of products given, the same analytes as that measured by the  
156 reference method, but which presents moreover, one or more criteria following: speed of  
157 analysis, easiness of execution and/or automation, analytical performances (limit of  
158 detection, specificity, etc). A specific guide for validation of alternative methods in the field of  
159 detection of antimicrobial residues in foodstuffs of animal origin was edited (Anon. 2005a). It  
160 defined the requirements relating to the organisation of preliminary and collaborative studies,  
161 carried out by one expert laboratory. This document established the general principle as well  
162 as the technical protocol for the validation of alternative methods in the field of detection of  
163 antimicrobial residues in foodstuffs of animal origin.

165 *Preliminary study*

166 The preliminary step has been divided in 3 parts.

167 *Step 1. Analysis of blank and spiked meat juice samples.* Porcine muscles came out of  
168 practice . The absence of antimicrobial substances in these pig samples, assumed as blank  
169 materials, was checked with the FPT and the STAR protocol. The negative results confirmed  
170 that these pig samples could be used to prepare blank meat juices.

171 Muscle samples were pressed with garlic press. Afterwards, meat juice samples were spiked  
172 with known concentrations of 6 different antimicrobials: sulfamethazine (sulphonamides),  
173 oxytetracycline (tetracyclines), tylosin (macrolides), amoxicillin (penicillins), ceftiofur  
174 (cephalosporins) and gentamycine (aminoglycosides). Each antimicrobial was spiked at 3  
175 concentrations: sulfamethazine (50, 100, 200  $\mu\text{g kg}^{-1}$ ), oxytetracycline (50, 100, 200  $\mu\text{g kg}^{-1}$ ),  
176 tylosin (50, 100, 200  $\mu\text{g kg}^{-1}$ ), amoxicillin (25, 50, 100  $\mu\text{g kg}^{-1}$ ), ceftiofur (100, 200, 400  $\mu\text{g kg}^{-1}$ )  
177 and gentamycine (50, 100, 200  $\mu\text{g kg}^{-1}$ ).

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

178 5 aliquots (5 combinations sample/antimicrobial/concentration) were prepared “blind to the  
179 technician” and analysed in duplicate only with the Premi®Test. The aliquots were stored in  
180 freezer (approximately -20°C) before analyses. Blank meat juice and spiked meat juice  
181 samples were analysed in duplicate only with the Premi®Test.

182  
183 *Step 2. Treatment of animals and analysis of incurred porcine muscle samples.* Two  
184 untreated pigs were slaughtered before the treatments of 3 other pigs. These pigs were  
185 supplied by a farm guaranteeing that the pigs did not receive antimicrobial treatments before.  
186 Moreover, the absence of antimicrobials in muscle samples was checked with the FPT and  
187 the STAR protocol.

188 Three pigs were treated: one with tylosin, one with amoxicillin and one with a mXture of  
189 oxytetracycline and sulfadimethoxine and slaughtered. Each material was analysed by a  
190 multi-residue LC/MS-MS method to quantify antimicrobials. The following concentrations  
191 were obtained: tylosin  $750.9 \pm 76.1 \mu\text{gkg}^{-1}$  (material 1), amoxicillin  $269.5 \pm 18.0 \mu\text{gkg}^{-1}$   
192 (material 2), oxytetracycline  $764.9 \pm 44.8 \mu\text{gkg}^{-1}$  and sulfadimethoxine  $151.1 \pm 13.2 \mu\text{gkg}^{-1}$   
193 (material 3).

194 Afterwards, 5 pieces of each material (blank and treated animals) of approximately 20g were  
195 (5 combinations sample/antimicrobial/concentration) prepared “blind to the technician” and  
196 analysed in duplicate with 3 methods: Premi®Test, STAR protocol and FPT. These samples  
197 were stored in freezer at approximately -20°C before analyses.

198  
199 *Step 3. Analysis of field samples.* This step allowed to compare Premi®Test, STAR protocol  
200 and FPT, on “naturally” incurred samples, for a wide number of antimicrobials and matrices  
201 of different origins (e.g. different species). The samples came from a pilot study concerning  
202 the implementation of a new screening method of the antimicrobial residues in meat (Anon.  
203 2005b). The samples were sent regularly to laboratories from the French veterinary services  
204 (official control). SX field veterinary laboratories (LVD or “Laboratoire Vétérinaire  
205 Départemental” in French) were trained to the implementation of the Premi®Test and one

1  
2  
3 206 plate *Bacillus cereus* (detection of tetracyclines) (STAR protocol) and analyzed 1427 field  
4  
5 207 samples, over a period of 4 months. All their positive samples (with Premi®Test and/or  
6  
7 208 *Bacillus cereus*) were sent frozen to the CRL, every 15 days. All the samples characteristics  
8  
9 209 (species, reception date, analysis date, sending date, ...etc...), as well as the results  
10  
11 210 obtained with Premi®Test and *Bacillus cereus*, were also sent to the CRL. All these samples  
12  
13 211 coming from the field laboratories where stored in the freezer at their arrival in our laboratory.  
14  
15 212 Then, blind analyses were carried out at the CRL with the Premi®Test, the FPT, and the  
16  
17 213 STAR protocol including the *Bacillus cereus* plate. Finally, the positive samples with at least  
18  
19 214 one of these methods were analyzed by the multi-residue LC/MS-MS method (systematic  
20  
21 215 screening, then identification and quantification). The doubtful samples with Premi®Test  
22  
23 216 were also tested by LC/MS-MS.  
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

#### 218 *Interlaboratory study*

219 11 laboratories have been contacted to participate to this inter-laboratory study, including the  
220 6 field laboratories which had already participated to the step 3 of the preliminary study.  
221 When an AFNOR validation is implemented, an expert laboratory is designated by the  
222 AFNOR Technical Office. The expert laboratory performed the intra-laboratory validation and  
223 organised the inter-laboratory study. Moreover, during the inter-laboratory study, the results  
224 of the expert laboratory are not included with all the participants' results, but these results are  
225 considered as the reference results (to be obtained by the participants). In this case, the  
226 Community Reference Laboratory was considered as the expert laboratory.  
227

228 Spiked muscle juice samples were used for the interlaboratory study, instead of incurred  
229 samples, for several reasons: Firstly, it is very difficult to prepare raw muscle with a target  
230 concentration of each antimicrobial because of the individual variability of animals'  
231 pharmacokinetic (depletion of the antibiotic in tissues). Moreover, the use of raw pieces of  
232 meat did not allow to mince meat to adjust the concentration. Secondly, the study was based  
233 on several animal species. Therefore, the treatment should have been performed in each

234 specie. This would have been very expensive, time consuming and impossible to be  
235 implemented in our laboratory. Thirdly, when producing incurred muscles and sending raw  
236 muscle samples (pieces of meat), it is very difficult to ensure the homogeneity of the  
237 samples, which is a basic condition when organising a collaborative study.

238  
239 *Preparation of the materials.* Porcine, bovine and chicken muscles were pressed. Blank meat  
240 juice samples (negative controls and blank unknown samples) were prepared. Furthermore,  
241 spiked samples were prepared with 4 different antibiotics, at 3 concentrations  
242 Oxytetracycline and ceftiofur were added to porcine meat juice samples, sulfamethazine to  
243 bovine meat juice samples and finally tylosin to chicken meat juice samples Table I presents  
244 the content of the 16 combinations.

245 “[Insert Table I about here]”

246 A stability study was carried out on materials before sending of the samples and over the  
247 period of analyses by the participants. The samples were analysed with the Premi®Test at  
248 the CRL. Stability of materials was proved over the period of analyses. A random codification  
249 of the materials was performed. Each laboratory was identified by a code (from A to P).

250  
251 *Sending of the materials.* One negative control from each species was sent and one positive  
252 control containing penicillin G at  $10 \mu\text{g kg}^{-1}$ . 32 different frozen meat juice samples (16  
253 materials in blind duplicate: spiked and blank samples) were sent under frozen conditions  
254 (dried ice) to the participants, in order to ensure stability of matrix and analyte. The samples  
255 were stored in freezer at their arrival. Eleven laboratories received with their parcel  
256 instructions and a results form. The participants had no information about the antibiotics  
257 contained in the materials.

258  
259 Because it is recommended to test one negative control from each analysed species, each  
260 laboratory received a table indicating the species of origin of each sample, according to  
261 sample code (1 to 32), in order to compare the results of each sample with the negative

control of the corresponding species. All analyses were carried out in blind duplicate (2 different series of analyses), with Premi®Test only. The analyses were performed within one week maximum after receiving the samples. A negative control for each species, a positive sample (spiked with penicillin G at 10 µg kg<sup>-1</sup>) and coded materials were analysed. All the results were returned rapidly and compiled at the CRL.

## Results and discussion

### *Preliminary study*

*Step 1. Analysis of blank and spiked meat juice samples.* Among the 20 blank meat juice samples analysed in duplicate, only one meat juice was positive twice and 4 other meat juices were "doubtful" on one of the two repetitions and negative on the other. 15 meat juices were negative twice. The false positive rate is the number of positive results for blank samples (free of antibiotic substances) divided by the total number of positive samples (the same number plus the number of contaminated samples showing positive results) and multiplied by 100. 18% ( $= 6/(6+27)*100$ ) of false positive results was observed. The result was satisfactory because the false positive rate should be minimal for a screening method, since the samples declared positive should be confirmed by a physicochemical method for identification and quantification.

The detection limit corresponded to the lowest concentration which gave a positive or a doubtful result for each of the 5 replicates. Table II presents the determination of limits of detection of Premi®Test for the 6 antimicrobials tested.

“[Insert Table II about here]”

The study of spiked pig meat juice samples showed that, for 5 molecules belonging to 4 different classes of antibiotics, the detection limit of Premi®Test was at the level of one or two MRL maximum. For only one antibiotic (gentamicin), the detection limit was higher than 2 times the MRL (40% of positive results at 2xMRL). The false negative rate corresponds to

1  
2  
3 290 the number of negative results obtained for contaminated samples (spiked samples) divided  
4  
5 291 by the total number of negative samples (the same number plus the number of blank  
6  
7 292 samples giving a negative result) and multiplied by 100. The false negative rate calculated at  
8  
9 293 1\*MRL was equal to 22 % ( = 10/(10+34)). However, the false negative rate calculated at  
10  
11 294 twice the MRL was equal to 8 % ( = 3/(3+34)).  
12  
13  
14 295

15  
16 296 *Step 2. Treatment of animals and analysis of incurred porcine muscle samples.* The  
17  
18 297 concentrations of naturally incurred samples were much higher than the respective MRL of  
19  
20 298 the 4 antimicrobials (from 1.5 to 7.6 times the MRL). However, the analyses of incurred  
21  
22 299 materials with known antimicrobial concentrations was of great interest. Table III summarises  
23  
24 300 the results of step 2.

301 "[Insert Table III about here]"  
302

303 The data exploitation was carried out according to the reference document of AFNOR  
304 validation (Anon. 2005a). The exploitation is based on the comparison of 2 methods: the  
305 reference method and the alternative method. The FPT was set as the reference method and  
306 the Premi®Test is the alternative method.  
307

308 Three different parameters were calculated which allowed to compare reference and  
309 alternative method: relative accuracy ( $AC = (PA + NA)/N \cdot 100\%$ ), relative specificity ( $SP =$   
310  $(NA/N_-) \cdot 100\%$ ), relative sensitivity ( $SE = (PA/N_+) \cdot 100\%$ ), where: NA is the negative  
311 agreement (negative result obtained with both methods), PA: the positive agreement  
312 (positive result obtained with both methods), ND: the negative discrepancy (positive result  
313 obtained with reference method and negative result with alternative test), PD: the positive  
314 discrepancy (negative result obtained with reference method and positive result with  
315 alternative test); N = NA + PA + PD + ND: total number of samples; N- is the total number of  
316 negative samples obtained with the reference method (NA + PD); N+ is the total number of  
317 positive samples obtained with the reference method (PA + ND).



1  
2  
3 318 The relative accuracy, the relative specificity and the relative sensitivity were calculated as  
4  
5 319 70%, 42.9% and 84.6% respectively. Therefore, the relative accuracy and specificity were  
6  
7 320 quite satisfactory. The low value of relative specificity could be explained because the  
8  
9 321 sensitivity of the FPT was sometimes insufficient for certain antibiotics.  
10  
11 322

12  
13  
14 323 In conclusion, FPT and Premi®Test gave concordant results when looking at the  
15  
16 324 performance characteristics (relative accuracy, the relative specificity and the relative  
17  
18 325 sensitivity). However, at this step, the false negative and the false positive rates were lower  
19  
20 326 for the Premi®Test (both 0 %) than for the FPT (33 and 40 % respectively).  
21  
22 327

23  
24  
25 328 *Step 3. Analysis of field samples.* 1427 field incurred samples, originated from 6 French field  
26  
27 329 veterinary laboratories, were analyzed implementing Premi®Test and *Bacillus cereus* plate.  
28  
29 330 1325 samples were detected negative and 102 samples doubtful (36) and positive (66) with  
30  
31 331 the Premi®Test.  
32  
33 332

34  
35 333 Furthermore, among the 1325 negative results, 10 samples were detected positive with the  
36  
37 334 *Bacillus cereus* plate. Therefore, 112 muscle samples were sent to the CRL to be confirmed.  
38  
39 335 The correlation between the Premi®Test results of LVD and AFSSA was studied (Table IV).  
40  
41 336 76% of the samples (78/102) found positive or doubtful with Premi®Test in the LVD, were  
42  
43 337 found positive or doubtful with Premi®Test at the CRL. 24 samples were positive or doubtful  
44  
45 338 at the LVD and negative at AFSSA. It could be due either to false positive results of the field  
46  
47 339 laboratories, or to antibiotic instability between the 2 analyses, although the best storage and  
48  
49 340 transport conditions have been strictly respected. Among the 10 samples negative with  
50  
51 341 Premi®Test and positive with *Bacillus cereus* in the field laboratories, 6 were found positive  
52  
53 342 or doubtful with Premi®Test at the CRL and confirmed positive with *Bacillus cereus*. The 4  
54  
55 343 other samples were found positive only on plate Bc6. Finally, the presence of a tetracycline  
56  
57 344 was confirmed by LC/MS-MS in 9 of these samples. Therefore, *Bacillus cereus* plate (one  
58  
59  
60

1  
2  
3 345 plate of the STAR protocol, which is selective for the detection of tetracyclines) is more  
4  
5 346 sensitive for tetracyclines than Premi®Test.

6  
7 347 “[Insert Table IV about here]”  
8

9  
10 348  
11 349 The false positive rate in field laboratories was sometimes high, mainly at the beginning of  
12  
13 350 the study. Afterwards, with the reading experience, most of the positive results with  
14  
15 351 Premi®Test at the field laboratories were confirmed positive with Premi®Test at the CRL.

16  
17 352 Many animal species were studied in step 3 because samples were from field laboratories  
18  
19 353 origin, while the 2 first steps were based only on pig muscle. The distribution of the samples  
20  
21 354 in the different species is presented (Table IV).  
22  
23 355

24  
25 356 Confirmatory rate of LC/MS-MS method (number of samples really containing antimicrobial  
26  
27 357 residues divided by the number of tested samples (positive screening) multiplied by 100) was  
28  
29 358 equal to 41% (25/61). The confirmatory rate varied between species. There were more false  
30  
31 359 positive results in some species (37 % for bovine samples instead of 75 % for poultry  
32  
33 360 samples).  
34  
35 361

36  
37 362 The results of the comparative analysis between the alternative method Premi®Test and the  
38  
39 363 reference method FPT are presented in Table V.

40  
41 364 “[Insert Table V about here]”  
42

43  
44 365  
45  
46 366 When the data analysis was based only on the 112 samples reanalysed in the expert  
47  
48 367 laboratory, relative accuracy (33.9%) and relative specificity (33.4%) were very low. Only  
49  
50 368 relative sensitivity (70 %) was satisfactory, as in the first step of the validation study.  
51  
52 369 However, in this case, Premi®Test negative samples (1315 negative samples / 1427  
53  
54 370 analyzed samples) obtained in the field laboratories were not taken into account. Only the  
55  
56 371 positive samples in the field laboratories were reanalysed at the CRL with Premi®Test and  
57  
58 372 FPT. None of the 1315 negative samples was analysed with the FPT. By experience (a  
59  
60



373 previous study in 2005) (data not shown), all the samples which were declared negative with  
374 Premi®Test and negative with *Bacillus cereus* were also all negative with the FPT (28  
375 samples). Therefore it was assumed that the 1315 samples, found negative with both  
376 Premi®Test and *Bacillus cereus*, would have also been negative with the FPT. In this case,  
377 the agreement (relative accuracy 94.8%, relative specificity 95%) between the results of  
378 Premi®Test and FPT was very satisfactory. The 2 methods were declared different by a  
379 statistical test, because of a high rate of positive deviation (Premi®Test + / FPT -) which may  
380 be caused partially by false positive results of the Premi®Test and/or by the lack of sensitivity  
381 of the reference method towards some antimicrobial residues.

382  
383 The agreement (relative accuracy) between the results of Premi®Test and STAR was equal  
384 to 42.99%, the relative specificity 31.3% and the relative sensitivity 71.9%. Therefore, it is  
385 quite similar to the comparison of FPT with Premi®Test. The agreement between the FPT  
386 and the STAR protocol was higher (70 %) (data not shown). Only one sample was negative  
387 with the STAR protocol and positive with the FPT. The absence of antimicrobial residues in  
388 this sample was confirmed by LC/MS-MS.

389  
390 The results obtained in the field laboratories, and then at the CRL, with the Premi®Test, the  
391 FPT, the STAR protocol and the confirmatory method by LC/MS-MS are compared (Table  
392 VI). The number of positive results with the Premi®Test (78) was much higher than the  
393 number of positive with the multi-plate tests (FPT 10; STAR 31 samples). Among the 112  
394 samples analysed at the CRL, only the positive or doubtful samples with one of the screening  
395 methods (Premi®Test, FPT, STAR) were confirmed by LC/MS-MS (88 samples).

396 “[Insert Table VI about here]”

397  
398 After the confirmatory analyses, 31 samples really contained antimicrobial residues (35% of  
399 the confirmed samples) at different levels (26 samples at concentrations lower than the  
400 respective MRLs and 5 only at concentrations higher than the respective MRLs).

1  
2  
3 401 The false positive rates of the FPT, the Premi®Test and the STAR protocol were 3%  
4  
5 402  $((1/(1+31))*100)$ , 62%  $((52/(52+31))*100)$  and 31 %  $((14/(14+31))*100)$  respectively. The false  
6  
7 403 positive (rate of each method was calculated as the number of positive results for blank  
8  
9 404 samples (free of antibiotic substances) divided by the total number of positive samples (the  
10  
11 405 same number plus the number of contaminated samples showing positive results) and  
12  
13 406 multiplied by 100.  
14

15  
16 407  
17  
18 408 52 samples were Premi®Test positive or doubtful, but no antibiotic residue could be  
19  
20 409 identified when analyzed by LC/MS-MS (). However, a false-positive result could be due to  
21  
22 410 the degradation of the antimicrobials initially contained in the sample (too long delay before  
23  
24 411 the analysis by LC/MS-MS, very unstable molecules...). Furthermore, it could be false  
25  
26 412 compliant results of the confirmatory method. The antimicrobial present in the sample could  
27  
28 413 not be detected and identified by the multi-residue LC/MS-MS method if the molecule is not  
29  
30 414 present in the spectrum of detection of the method (metabolites, ...). or the sensitivity could  
31  
32 415 be insufficient.  
33

34  
35 416  
36  
37 417 After a positive result with Premi®Test, the presence of antimicrobial residues was  
38  
39 418 confirmed in 26 samples (33 %), from which 4 samples contained antimicrobials at  
40  
41 419 concentrations upper than the MRL. Three of the 10 positive FPT samples and 4 of the  
42  
43 420 STAR positive samples were at concentrations upper than the MRL. Premi®Test and STAR  
44  
45 421 screened positive 22 and 13 samples respectively which really contained antimicrobial  
46  
47 422 compounds at concentrations lower than MRL. The FPT detected only 6 of these samples.  
48  
49 423 Therefore, the sensitivity of Premi®Test and STAR protocol was lower than those of the FPT.  
50  
51 424 The highest confirmatory rate was obtained for the FPT (90 %). The false negative rates of  
52  
53 425 the FPT, the Premi®Test and the STAR protocol were equal to 8 %, 28 % and 20 %  
54  
55 426 respectively. The false negative rate was calculated as the number of false negative results  
56  
57 427 divided by the total of the true negative samples plus the number of false negative results for  
58  
59 428 each method.  
60

1  
2  
3 429 The most commonly detected antimicrobial classes detected during this pilot study were  
4  
5 430 beta-lactams (penicillin and amoxicillin) (15 samples) and tetracyclines (doxycycline,  
6  
7 431 tetracycline, oxytetracycline and chlortetracycline) (15 samples). Premi®Test (12 positive  
8  
9 432 samples) was much more sensitive for the detection of beta-lactams than the FPT (1 positive  
10  
11 433 sample) and the STAR protocol (5 positive samples), even too sensitive in some cases (10  
12  
13 434 positive samples confirmed at concentrations lower than MRL). Premi®Test was also more  
14  
15 435 sensitive for the screening of sulphonamides than the 2 other methods (FPT and STAR failed  
16  
17 436 to detect 1 sample at a concentration higher than the MRL). Finally, Premi®Test was less  
18  
19 437 sensitive than the FPT and especially than the STAR protocol for the detection of  
20  
21 438 tetracyclines. Therefore, this study demonstrated the ability of Premi®Test to detect samples  
22  
23 439 at the MRL level for sulphonamides and beta-lactams, but not for tetracyclines. These  
24  
25 440 conclusions confirmed the results obtained by different teams during the past years  
26  
27 441 ((Reybroeck 2000a, (Reybroeck 2000b, Okerman *et al.* 2004, Popelka *et al.* 2005).  
28  
29 442  
30  
31 443 Table VII presented the combined results of a previous study realized in 2003 and 2004 (64  
32  
33 444 samples really containing antimicrobial residues) with the pilot study.  
34  
35 445  
36  
37 446  
38  
39 447  
40  
41 448  
42  
43 449  
44  
45 450  
46  
47 451  
48  
49 452  
50  
51 453  
52  
53 454  
54  
55  
56  
57  
58  
59  
60

“[Insert Table VII about here]”

447 After the confirmatory analyses of positive samples, 31 samples contained antimicrobial  
448 residues at concentrations lower than the respective MRLs and 33 samples at concentrations  
449 higher than the respective MRLs. The total number of positive samples reported with  
450 Premi®Test (52) was higher than with FPT (38). However, the number of samples detected  
451 positive with Premi®Test (26) or FPT (25) which really contained antimicrobial compounds at  
452 concentrations higher than MRL was identical. Moreover, 6 false negative results were  
453 obtained with Premi®Test and 7 with FPT.

1  
2  
3 455 The presence of beta-lactams was confirmed in 17 samples by the LC/MS-MS method. All of  
4  
5 456 these 17 samples were detected positive or doubtful samples with Premi®Test, while only 7  
6  
7 457 samples were detected positive with the FPT (1<MRL and 6> MRL). Therefore, the detection  
8  
9 458 capability of Premi®Test for beta-lactams was better than FPT (). The sensitivity was also  
10  
11 459 lower for sulphonamides (4 positive samples Premi®Test, 4 negative samples FPT). Two of  
12  
13 460 these samples really contained sulphonamides at concentrations higher than the MRL and  
14  
15 461 the FPT failed to detect them while the Premi®Test succeeded to detect. However, the  
16  
17 462 sensitivity of FPT for tetracyclines was better (15 doubtful or positive samples with  
18  
19 463 Premi®Test instead of 19 positive samples with FPT).  
20  
21  
22  
23

24  
25 464  
26  
27 465 The most detected antimicrobials were tetracyclines (25 samples) and beta-lactams (17  
28  
29 466 samples). Then, macrolides (6), sulphonamides (4), quinolones (3) or mix of antimicrobials  
30  
31 467 (8) were found.  
32  
33 468

#### 34 469 *Collaborative study*

35 470 *Results of the expert laboratory.* The expert laboratory obtained only one positive false result  
36  
37 471 for the 2 series of analyses for the white chicken sample (code 10), whereas the same  
38  
39 472 sample (code 27) was found negative for the 2 series. The L1 concentration was selected to  
40  
41 473 give negative results (Table I). The expert laboratory did not obtain any false negative result  
42  
43 474 because the L3 concentration was that designed to give positive results, whereas the L2  
44  
45 475 concentration was to be at the limit of sensitivity of the test.  
46  
47  
48  
49

50  
51 477 It should be noted that the later analysis of the results was carried out while taking as  
52  
53 478 principle that the doubtful results are positive results, this which is applied at the time for  
54  
55 479 routine analyses.  
56

57 480 “[Insert Table VIII about here]”  
58  
59 481  
60

1  
2  
3 482 The results of the expert laboratory were very satisfactory (Table VIII): 14 blank samples out  
4  
5 483 of 16 were negative (only 2 positive results for chicken samples) (L0). Below the assumed  
6  
7 484 limit of detection, 14 samples out of 16 were negative (L1). At the assumed limit of detection,  
8  
9 485 all the samples were positive (L2). Above the assumed limit of detection, all the samples  
10  
11 486 were positive (L3).  
12  
13

14 487  
15  
16 488 *Results of the participants.* Before the study, the expert laboratory stated that the results of  
17  
18 489 one laboratory would be removed when: the negative control was detected positive, the  
19  
20 490 positive control was detected negative, if samples were in bad condition at the reception, if a  
21  
22 491 problem of storage was established. Two laboratories were finally eliminated from the  
23  
24 492 analysis of the results: Laboratory L for several reasons: the parcel delivery was delayed,  
25  
26 493 samples were defrosted and chicken negative controls were detected positive for the 2 series  
27  
28 494 of analyses; Laboratory H because chicken negative controls were detected positive for 1st  
29  
30 495 series of analyses. The raw data of the participants for the 16 combinations are presented in  
31  
32 496 Table IX. Each participant received 32 samples (16 materials in blind duplicate) that they  
33  
34 497 have analysed in 2 different series.  
35  
36

37 498 “[Insert Table IX about here]”  
38  
39 499

40 500 The results were homogeneous between laboratories. The results were analysed and  
41  
42 501 summarised in Table X. Concerning negative samples (L0) (16 by laboratories corresponding  
43  
44 502 to 3 different species), 4 laboratories found 100% of negative results, 5 laboratories found 1  
45  
46 503 or 2 samples doubtful or positive, sometimes in only one series of analyses (laboratories M  
47  
48 504 and N).  
49  
50

51 505 “[Insert Table X about here]”  
52  
53 506

54  
55  
56  
57 507 For the samples containing antibiotics at a concentration below the detection limit (L1), 6  
58  
59 508 laboratories found 100% of negative results; 3 laboratories found 1 or 2 samples which were  
60  
509 doubtful or positive. When the concentration was considered near the detection limits (L2),

1  
2  
3 510 tylosin was generally detected (83 % of positive results), whereas the other antibiotics were  
4  
5 511 less detected (oxytetracycline 11%, ceftiofur 11% and sulfamethazine 17%).  
6  
7  
8

9  
10 513 The average rate of positive samples to the L3 concentration was 81 %. The rate of detection  
11  
12 514 to the L3 concentration is 100% for the 4 antibiotics and for 3 laboratories (B, D, N). This rate  
13  
14 515 was 100% for the totality of the laboratories concerning tylosin and ceftiofur.  
15  
16

17 516  
18 517 The results of the participating laboratories were analyzed in order to calculate different  
19  
20 518 validation parameters. A laboratory was removed from the analysis because due to a delay  
21  
22 519 in transport the samples arrived in defrosted conditions.  
23  
24

25 520  
26 521 Table X presents also the analysis of the results of all the participating laboratories in term of  
27  
28 522 reproducibility, by material (combination animal/antibiotic species) and in a global way. The  
29  
30 523 reproducibility, expressed as a percentage, is the ratio of the number of identical results, the  
31  
32 524 most common type (e.g. negative results for blank samples or positive result for samples  
33  
34 525 contaminated with an antimicrobial concentration exceeding the detection limit) on the total  
35  
36 526 number of analyses.  
37  
38

39 527  
40 528 The results of the participating laboratories in term of reproducibility are very satisfactory with  
41  
42 529 an average percentage of 89,1%. The worse reproducibility was observed for the  
43  
44 530 combination bovine/sulfamethazine. A little higher concentration of sulfamethazine would  
45  
46 531 undoubtedly have given better results in term of reproducibility.  
47  
48  
49

50 532  
51 533 The percentage of specificity SP for the levels L0 and L1 was calculated as:  $SP = [1 - (FP/N_-) * 100\%]$   
52  
53 534 where:  $N_-$ : total number of tests L0 and L1 and FP: number of false positive results.  
54  
55 535 The percentage of sensitivity SE for each positive contamination level L2 and L3 was  
56  
57 536 calculated using the following equation:  $SE = (TP/N_+) * 100\%$  where:  $N_+$ : total number of  
58  
59 537 tests L2 or L3 respectively and TP: a number of true positive.  
60

538 Specificity (95,3 %) and sensitivity of the Premi®Test to the level L3 (72,5 %) were very  
539 satisfactory.

540  
541 The repeatability was estimated in each laboratory: 1- by comparing the results of the 2 tests  
542 performed on each sample (2 different sets of analyses), knowing that the knowledge of the  
543 initial result can influence the reading at the second analysis, 2- by comparing the results  
544 obtained with the 2 samples of each pair. The repeatability expressed as a percentage, is the  
545 ratio of the number of identical results per couple of analyses on the total number of couples.  
546 The following table presents the total analysis of the results of all the participants obtained  
547 with the Premi®Test in term of repeatability. (Tables XI).

548 “[Insert Table XI about here]”

549  
550 The results of the participating laboratories in term of repeatability are very satisfactory with  
551 an average percentage of 94,8% for the same sample and 92,7% per 2 identical samples  
552 (pair). The limits of detection of the Premi®Test during the preliminary study were confirmed  
553 by the collaborative study.

## 554 555 **Conclusion**

556 This paper presents a significant dataset concerning the performance of the Premi®Test, for  
557 different kind of samples: spiked meat juice samples, incurred samples, routine field samples  
558 (confirmed by a LC/MS-MS method). The detection capabilities of Premi®Test for beta-  
559 lactams (amoxicillin, ceftiofur), one macrolide (tylosin) and tetracycline were at the level of  
560 the respective Maximum Residue Limits (MRL) in muscle samples or even lower. The  
561 applicability of the test to different animal species was proven. Moreover, the applicability of  
562 Premi®Test to routine analysis of samples was demonstrated. The Four Plate Test  
563 (reference method) and the Premi®Test showed comparable performances in term of  
564 sensitivity and specificity. Moreover, the false negative rate of Premi®Test was always lower  
565 than that of the FPT. This is the most important parameter to minimise for a screening



1  
2  
3 566 method for antibiotic residues. On the contrary, the false positive rate of Premi®Test  
4  
5 567 appeared, in step 3, higher than that of the FPT. This means that the number of samples to  
6  
7 568 confirm by physico-chemical methods would be higher if the laboratories used the  
8  
9 569 Premi®Test alone. However, it means also that more real positive samples would be  
10  
11 570 detected because Premi®Test was more sensitive than FPT for some antimicrobials (beta-  
12  
13 571 lactams and sulphonamides). Beta-lactams and some sulphonamides were satisfactorily  
14  
15 572 detected at the MRL level by the Premi®Test.  
16  
17  
18  
19

20  
21 574 This is the first time to our knowledge that an interlaboratory study organised for the  
22  
23 575 detection of antibiotics with Premi®Test was described. The results of the collaborative study  
24  
25 576 were very satisfactory. The results of 9 laboratories were finally analyzed, plus the expert  
26  
27 577 laboratory. Specificity was estimated at 95.3 %, moreover the sensitivity of the test to the L3  
28  
29 578 level was calculated to 72,5%. These results were similar with those obtained at the time of  
30  
31 579 preceding validations of kits of detection of the antibiotic residues in milk. The results in term  
32  
33 580 of repeatability and reproducibility are very satisfactory, with average percentages of 94,8%  
34  
35 581 and 92,7 % for the repeatability and of 89,1 for reproducibility.  
36  
37  
38  
39

40  
41 583 In conclusion, Premi®Test is easy to perform. It is ideal for an "on site" use  
42  
43 584 (slaughterhouses, test laboratories) as no special laboratory equipment is needed to perform  
44  
45 585 the test. The rapidly response "yes/no" result is simply read by colour comparison.  
46  
47 586 Premi®Test is applicable to the muscles of various species (porcine, bovine, ovine...), by  
48  
49 587 using as negative control a "blank" muscle of each analyzed specie to optimise the reading  
50  
51 588 time.  
52

53  
54  
55 590 Finally, since 2006, the field laboratories in France are authorised to use the Premi®Test as  
56  
57 591 a pre-screening test. All positive samples with Premi®Test are then mandatory analysed by  
58  
59 592 the FPT (Anon. 2006). All positive samples with the FPT are sent to our laboratory for  
60  
593 confirmation as usual.



1  
2  
3 594 **Acknowledgements**  
4

5 595 The authors thank Annie Rault, Anne de Courville, Sophie Gautier and Marie-Pierre  
6  
7 596 Fourmond for their technical participation to the preliminary study and Catherine Creff-Froger  
8  
9  
10 597 responsible of the Four Plate Test analyses. We would like to thank also Jean-Michel Fabre  
11  
12 598 (Phylum, Labège, France) for its help to the data analysis. Thanks to Françoise Goeijen  
13  
14 599 (DSM, The Netherlands), and to the 6 French field veterinary laboratories (LVD) which  
15  
16 600 participated to the preliminary study. Thanks finally to all the participants of the collaborative  
17  
18 601 study.  
19  
20  
21 602  
22  
23 603  
24  
25 604

605 **References**

- 606 ANON., 2005a, AFNOR Document, "Requirements relating to the preliminary and  
607 collaborative studies undertaken by an expert laboratory/application to the detection  
608 methods of antimicrobial residues and another molecules for related purpose" revision 0.
- 609 ANON., 2005b, Memorandum DGAL/SDRRCC/N2005-8217 of the 08/09/2005, « New  
610 method of analysis for the research of antimicrobial residues in meat with Premi®Test and  
611 *Bacillus cereus*: implementation of a pilot action ». <http://agriculture.gouv.fr/>
- 612 ANON., 2006, Memorandum DGAL/SDRRCC/N2006-8240 of the 04/10/2006, « Control plan  
613 for antimicrobial residues in slaughter meats, poultry, game, rabbits and breeding fish ».  
614 <http://agriculture.gouv.fr/>
- 615 ARTS, C. J. M., GEIJP, E., STARK, J., WITKAMP, R., 2000, Premi®Test: a broad spectrum  
616 screening test for detection of antimicrobial compounds in meat, organs and urine. In:  
617 *Proceedings of the EuroResidue IV Conference* (Veldhoven, The Netherlands), Van  
618 Ginkel LA, Ruiter A., 186-192.
- 619 ARTS, C. J. M. and WITKAMP, R. F., 1999, The Premi Test for screening for residues of  
620 antimicrobial compounds in meat, A summary report. TNO nutrition and Food Research  
621 Institute, TNO project number 50.736/01.01 (The Netherlands).
- 622 BOGAERTS, R., WOLF, F., 1980, A standardized method for the detection of residues of  
623 antibacterial substances in fresh meat. *Die Fleischwirtschaft*, **60**, 672-674.
- 624 CANTWELL, H. and O'KEEFFE, M. 2006, Evaluation of the Premi®Test and comparison  
625 with the One Plate Test for the detection of antimicrobial substances in kidney. *Food*  
626 *Additives and Contaminants*, **23** (2), 120-125.
- 627 Council Regulation (EEC) No. 2377/90. 26 June 1990. Laying down a Community procedure  
628 for the establishment of maximum residue limits of veterinary medicinal products in  
629 foodstuffs of animal origin.
- 630 FABRE, J.M., 2003, Des Méthodes de recherche d'antibiotiques dans la viande se  
631 développent en France. *La Semaine Vétérinaire*, July 2003.

- 1  
2  
3 632 FABRE, J.M., 2003, Recherche des résidus d'antibiotiques dans la viande: De nouvelles  
4 méthodes pour de nouveaux besoins. *La Semaine Vétérinaire* July 2003  
5  
6 633  
7 634 FABRE, J.M., MIRCOVICH, C., GEIJP, E., 2004, Antibiotic residues in pork and poultry meat  
8  
9 635 in France: the current situation and an evaluation of a new screening test. *Bulletins des*  
10  
11 636 *GTV*, **23**, 305-309.  
12  
13 637 GAUDIN, V., MARIS, P., FUSELIER, R., RIBOUCHON, J.L., CADIEU, N., RAULT, A., 2004,  
14  
15 638 Validation of a microbiological method: the STAR protocol, a five-plate test, for the  
16  
17 639 screening of antibiotic residues in milk. *Food Additives and Contaminants*, **21**, 422-433.  
18  
19 640 OKERMAN, L., CROUBELS, S., CHERLET, M., DE WASCH, K., DE BACKER, P., VAN  
20  
21 641 HOOFF, J., 2004, Evaluation and establishing the performance of different screening tests  
22  
23 642 for tetracycline residues in animal tissues. *Food Additives and Contaminants*, **21**, 145–  
24  
25 643 153.  
26  
27 644 POPELKA, P., NAGY, J., GERMUSKA, R., MARCINCAK, S., JEVINOVA, P., DE RIJK, A.,  
28  
29 645 2005, Comparison of various assays used for detection of beta-lactam antibiotics in  
30  
31 646 poultry meat. *Food Additives and Contaminants*, **22**, 557-562.  
32  
33 647 REYBROEK, W., 2000a, Performance of the Premi®Test using naturally contaminated meat.  
34  
35 648 In: *Proceedings of the EuroResidue IV Conference* (Veldhoven, The Netherlands), Van  
36  
37 649 Ginkel LA, Ruiters A., 909-912.  
38  
39 650 REYBROEK, W., 2000b, Detection of residues of antibiotics in foodstuffs with microbiological  
40  
41 651 tests using bacillus. *Proceedings of the Bacillus symposium*; 2000 August 30-31; Brugge,  
42  
43 652 Belgium.  
44  
45 653 STEAD, S., SHARMAN, M., TARBIN, J.A., GIBSON, E., RICHMOND, S., STARK, J., GEIJP,  
46  
47 654 E., 2004, Meeting maximum residue limits: an improved screening technique for the rapid  
48  
49 655 detection of antimicrobial residues in animal food products. *Food Additives and*  
50  
51 656 *Contaminants*, **21**, 216-221.  
52  
53  
54  
55  
56  
57  
58  
59 658  
60 659  
660

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

661  
662  
663  
664  
665  
666  
667  
668  
669  
  
670

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

671

For Peer Review Only

1 **Table I. Interlaboratory study: Content of the 16 materials sent in blind duplicate.**

		Porcine	Bovine	Porcine	Chicken
Antimicrobial	Level of contamination	Oxytetracycline	Sulfamethazine	Ceftiofur	Tylosin
MRL	/	100	100	1000	100
Spiked concentrations (µg/kg)	L0	'Blank'	'Blank'	'Blank'	'Blank'
	L1	20	20	40	10
	L2	200	200	400	100
	L3	400	400	800	200

2

3

4 **Table II. Results of the Premi®Test analyses on spiked juice samples (step 1).**

Antibiotic family	SULPHONAMIDE	TETRACYCLINE	MACROLIDE	BETA-LACTAM	AMINOGLYCOSIDE	BETA-LACTAM	
Antibiotic	Sulfamethazine	Oxytetracycline	Tylosin	Amoxicillin	Gentamicin	Ceftiofur	<b>Global</b>
MRL(muscle) ( $\mu\text{g kg}^{-1}$ )	100	100	100	50	50	1000	/
Tested concentrations ( $\mu\text{g kg}^{-1}$ )	50/ <b>100</b> /200	50/ <b>100</b> /200	50/ <b>100</b> /200	25/ <b>50</b> /100	<b>50</b> /100/200	100/200/400	/
Detection rate at 0.5 MRL	0%	60%	80%	100%	(0%)*	-	<b>48%</b>
Detection rate at MRL	20%	80%	100%	100%	0%	(100%)	<b>67%</b>
Detection rate at 2* MRL	100%	100%	100%	100%	40%	(100%)	<b>90%</b>
<b>Detection capability</b>	<b>2xMRL</b>	<b>2xMRL</b>	<b>MRL</b>	<b>0.5xMRL</b>	<b>&gt; 2xMRL</b>	<b>0.5xMRL</b>	/

7 \*MRL in bold character

9 \* The percentage is between brackets when there were no analyses performed at this level of  
 10 concentration. i.e. All the tested concentrations for ceftiofur were below MRL and even 0.5 MRL. At  
 11  $400 \mu\text{g kg}^{-1}$ , 100 % of the results were positive or doubtful.

12 Table III. Analyses of naturally incurred samples with the 3 screening methods (step 2).

13

Antibiotic	OTC/SDMX	Amoxicillin	Tylosin	Global results of incurred samples	Blank	Fp+	Fp-
MRL (pig muscle) ( $\mu\text{g}/\text{kg}$ )	100 / 100	50	100				
Concentrations by LC/MS-MS ( $\mu\text{g}/\text{kg}$ )	760 / 150	270	750				
Number of positive results with Premi <sup>®</sup> Test	5 / 5	5 / 5	5 / 5	15 / 15	0 / 5	0 %	0 %
Number of positive results with STAR	5 / 5	5 / 5	5 / 5	15 / 15	3 / 5	60 %	0 %
Number of positive results with FPT	5 / 5	4 / 5	1 / 5	10 / 15	2 / 5	40 %	33 %

14 *Fp+*: false positive rate; *fp-*: false negative rate; OTC: Oxytetracycline; SDMX: sulfadimethoxine

15



16 Table IV. Correlation between field laboratories and AFSSA, comparison of the 3 screening  
 17 tests and LC/MS-MS method in relation to the species (step 3).

18

	Specie	Bovine	Porcine	Poultry	Others	Unknown	TOTAL
<b>LVD</b>	Number of samples analysed at LVD <sup>b</sup>	379	671	205	26	146	<b>1427</b>
	Number of positive and doubtful results at LVD Premi®Test	63	26	1	5	7	102
<b>AFSSA</b>	Number of samples analysed at AFSSA	65	31	4	5	7	<b>112<sup>a</sup></b>
	Number of positive and doubtful results with Premi®Test	45	19	2	5	7	78
	Correlation LVD/AFSSA (%)	69	61	50	100	100	76
	Number of positive samples with FPT	3	5	2	0	0	10
	Number of positive samples with STAR	13	13	4	0	2	32
<b>AFSSA LC/MS-MS</b>	Number of tested samples	38	18	4	1	0	61
	Number of positive samples (identified molecule)	14	8	3	0	0	25
	Rate of positive confirmation (%)	37	44	75	0	/	41

19

20 <sup>a</sup> Total of the samples sent to AFSSA for confirmation

21 <sup>b</sup> LVD means "Laboratoire Vétérinaire Départemental" in French which is a field veterinary laboratory.

22

23 Table V. Correlation between the Premi®Test and the FPT (step 3).

	1st analysis (112 samples)	2 <sup>nd</sup> analysis (1427 samples)
Relative accuracy AC (%)	33.9	94.8
Relative specificity SP (%)	30.4	95.0
Relative sensitivity SE (%)	70.0	70.0

24 *Relative accuracy: AC = (PA + NA)/N\*100%*

25 *Relative specificity: SP = (NA/N-)\*100%*

26 *Relative sensitivity: SE = (PA/N+)\*100%*

27 *Where: NA is the negative agreement, PA: the positive agreement, ND: the negative discrepancy, PD: the*  
 28 *positive discrepancy;*

29 *N = NA + Pa + PD + ND: total number of samples*

30 *N- is the total number of negative samples obtained with the reference method (NA + PD)*

31 *N+ is the total number of positive samples obtained with the reference method (PA + ND)*

32

33 Table VI. Results of the identification of positive samples with LC/MS-MS at the AFSSA:  
 34 Comparison of Premi®Test, Four Plate Test and STAR protocol in 2005 (step 3).

35

Identified AB family	Quantification / MRL	Number of analysed	Premi®Test results			FPT results		STAR results	
			-	D	+	-	+	-	+
MACRO	<MRL	1		1			1		1
SULFA	<MRL	1		1		1		1	
TTC	<MRL	10	4	3	3	6	4	2	8
TTC	>MRL	2	1		1		2		2
BL	<MRL	10		2	8	10		7	3
BL	>MRL	2		2		1	1		2
TTC + SULFA	<MRL	1		1			1		1
BL + sulfa	>MRL	1		1		1		1	
MACRO + TTC + BL	<MRL	1		1		1		1	
TTC + BL	<MRL	1		1		1		1	
AMINO + BL	<MRL	1		1		1		1	
<b>Global</b>	<LMR	26	4	7	15	20	6	13	13
	>LMR	5	1	0	4	2	3	1	4
	<b>Total</b>	<b>31</b>	<b>5</b>	<b>7</b>	<b>19</b>	<b>22</b>	<b>9</b>	<b>14</b>	<b>17</b>
Absence	/	57	5	8	44	56	1	43	14
Not analysed	/	24	24			24		24	
<b>TOTAL</b>	/	<b>112</b>	<b>34</b>	<b>15</b>	<b>63</b>	<b>102</b>	<b>10</b>	<b>81</b>	<b>31</b>

36

37

38 Table VII. Results of the identification of positive samples with LC/MS-MS at the AFSSA:  
 39 Comparison of alternative method (Premi®Test) and reference method (FPT) from 2003 to  
 40 2005 (step 3).

41

Identified AB family	Quantification / MRL**	Number of analysed samples	Premi®Test results			FPT results	
			-	D*	+	-	+
Beta-lactams	<MRL	9	/	1	8	8	1
	>MRL	8	/	/	8	2	6
	<b>Sum***</b>	<b>17</b>					
Tetracyclines	<MRL	13	5	4	4	6	8
	>MRL	12	5	1	6	/	11
	<b>Sum</b>	<b>25</b>					
Sulphonamides	<MRL	2	/	1	1	2	/
	>MRL	2	/	/	2	2	/
	<b>Sum</b>	<b>4</b>					
Quinolones	>MRL	3	1	/	2	/	3
BL + Sulfa	<MRL	2	/	/	2	1	1
	>MRL	2	/	/	2	2	/
	<b>Sum</b>	<b>4</b>					
Tetra + Sulfa	<MRL	1	/	/	1	/	1
	>MRL	3	/	/	3	/	3
	<b>Sum</b>	<b>4</b>					
Tetra + BL	<MRL	1	/	/	1	1	/
Macrolides	<MRL	3	1	1	1	1	2
	>MRL	3	/	/	3	1	2
	<b>Sum</b>	<b>6</b>					
Global	<MRL	31	6	7	18	19	13
	>MRL	33	6	1	26	7	25
<b>TOTAL</b>	/	<b>64</b>	<b>12</b>	<b>8</b>	<b>44</b>	<b>26</b>	<b>38</b>

42 \* D: Doubtful

43 \*\* MRL: Maximum Residue Limit

44 \*\*\* Sum of samples confirmed lower and higher than respective MRLs.

45

46 **Table VIII. Results of the expert laboratory (AFSSA) during the interlaboratory**  
47 **study.**

48

Level of contamination	Oxytetracycline Porcine	Sulfamethazine Porcine	Ceftiofur Bovine	Tylosin Chicken
L0	0*	0	0	2
L1	0	0	0	2
L2	4	4	4	4
L3	4	4	4	4

49 *\* number of positive results*

50

51 **Table IX. Raw data of the participants for each material and the 16 combinations.**

Porcine Oxytetracycline																	
		L0 (0)				L1 (20*)				L2 (200)				L3 (400)			
		Mat 1		Mat 5		Mat 2		Mat 6		Mat 3		Mat 7		Mat 4		Mat 8	
Lab		A1	A2	A1	A2	A1	A2	A1	A2	A1	A2	A1	A2	A1	A2	A1	A2
A		-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	D
B		-	-	-	-	-	-	-	-	-	-	-	-	+	+	+	+
C		-	-	-	-	-	-	-	-	-	-	-	-	+	+	+	+
D		-	-	-	-	-	-	-	-	-	-	-	-	+	+	+	+
E		-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
F		-	-	-	-	-	-	-	-	+	+	+	+	+	+	+	+
G		-	-	-	-	-	-	-	-	-	-	-	-	D	D	D	D
M		-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
N		-	-	-	-	-	-	-	-	-	-	-	-	+	+	+	+
Porcine ceftiofur																	
		L0 (0)				L1 (40)				L2 (400)				L3 (800)			
		Mat 9		Mat 13		Mat 10		Mat 14		Mat 11		Mat 15		Mat 12		Mat 16	
Lab		A1	A2	A1	A2	A1	A2	A1	A2	A1	A2	A1	A2	A1	A2	A1	A2
A		-	-	-	-	-	-	-	-	-	D	-	-	+	D	+	+
B		-	-	-	-	-	-	-	-	-	-	-	-	+	+	+	+
C		-	-	+	+	-	-	-	-	-	-	-	-	+	+	+	+
D		-	-	-	-	-	-	-	-	-	-	-	-	+	+	+	+
E		-	-	-	-	-	-	-	-	-	-	-	-	D	D	+	+
F		D	D	D	D	-	-	D	D	-	D	+	+	+	+	+	+
G		-	-	-	-	-	-	-	-	-	-	-	-	+	+	+	+
M		-	-	-	-	-	-	-	-	-	-	-	-	+	+	+	+
N		-	-	-	-	-	-	-	-	-	-	-	-	+	+	+	+
Bovine sulfamethazine																	
		L0 (0)				L1 (20)				L2 (200)				L3 (400)			
		Mat 17		Mat 21		Mat 18		Mat 22		Mat 19		Mat 23		Mat 20		Mat 24	
Lab		A1	A2	A1	A2	A1	A2	A1	A2	A1	A2	A1	A2	A1	A2	A1	A2
A		-	-	-	-	-	-	-	-	-	-	-	-	D	-	-	-
B		-	-	-	-	-	-	-	-	-	-	-	-	+	+	+	+
C		-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
D		-	-	-	-	-	-	-	-	-	D	-	D	+	+	+	+
E		-	+	-	+	-	+	-	+	-	+	-	+	-	+	-	+
F		-	-	-	-	-	-	-	-	-	-	+	+	-	-	D	D
G		-	-	-	-	-	-	-	-	-	-	-	-	-	-	D	D
M		-	D	-	-	-	-	-	D	-	-	-	-	-	-	-	-
N		+	-	-	-	-	-	-	-	-	-	-	-	D	+	D	+
Chicken Tylosin																	
Conc		L0 (0)				L1 (10)				L2 (100)				L3 (200)			
		Mat 25		Mat 29		Mat 26		Mat 30		Mat 27		Mat 31		Mat 28		Mat 32	
Lab		A1	A2	A1	A2	A1	A2	A1	A2	A1	A2	A1	A2	A1	A2	A1	A2
A		-	-	-	-	-	-	-	-	+	D	-	D	D	+	D	+
B		-	-	-	-	-	-	-	-	+	+	+	+	+	+	+	+
C		-	-	-	-	-	-	-	-	+	+	+	+	+	+	+	+
D		-	-	-	-	-	-	-	-	+	+	+	+	+	+	+	+
E		-	-	-	-	-	-	-	-	-	-	-	D	+	+	+	+
F		-	-	-	-	-	-	-	-	+	+	+	+	+	+	+	+
G		-	-	-	-	-	-	-	-	+	+	-	D	+	+	+	+
M		-	-	-	-	-	-	-	-	D	-	D	D	+	+	+	+
N		-	-	-	-	-	-	-	-	D	+	+	+	+	+	+	+

52 *A1: first analysis; A2: second analysis; Mat: Material; \*Conc: concentrations (ng ml<sup>-1</sup>).*

53 **Table X. Analysis of the results of all the participating laboratories in term of**  
 54 **percentages of positive results for each material and in term of reproducibility, by**  
 55 **material (combination animal/antibiotic species) and global reproducibility.**

56

Material	Number of sample (number of the pair)	Level of contamination	False positive and true positive rates	Percentages of positive results for each material	Reproducibility (%)
Porcine OTC	1	<b>L0</b>	FP0 a)*	0%	100.0
	2	<b>L1</b>	FP1 b)	0%	100.0
	3	<b>L2</b>	TP2 c)	11%**	88.9***
	4	<b>L3</b>	<b>TP3 d)</b>	<b>69%</b>	69.4
Porcine ceftiofur	5	<b>L0</b>	FP0 a)	11%	83.3
	6	<b>L1</b>	FP1 b)	8%	94.4
	7	<b>L2</b>	TP2 c)	17%	88.9
	8	<b>L3</b>	<b>TP3 d)</b>	<b>53%</b>	100.0
Bovine sulfamethazine	9	<b>L0</b>	FP0 a)	17%	91.7
	10	<b>L1</b>	FP1 b)	6%	91.7
	11	<b>L2</b>	TP2 c)	11%	83.3
	12	<b>L3</b>	<b>TP3 d)</b>	<b>100%</b>	52.8
Chicken Tylosin	13	<b>L0</b>	FP0 a)	0%	100.0
	14	<b>L1</b>	FP1 b)	0%	100.0
	15	<b>L2</b>	TP2 c)	83%	83.3
	16	<b>L3</b>	<b>TP3 d)</b>	<b>100%</b>	100.0
<b>Total</b>					<b>89.1</b>

57

58

59 *FP : False positive rate*60 *TP : True positive rate*61 *a) False positive at level L<sub>0</sub>*62 *b) False positive at level L<sub>1</sub>*63 *c) True positive at level L<sub>2</sub>*64 *d) True positive at level L<sub>3</sub>*65 *\* At each level of contamination, 9 laboratories reported 4 results for 2 materials in blind duplicate. The total number of samples per level is 9\*4 = 36 samples.*66 *\*\* TP2 c) = 4 TP / 36 samples at level L<sub>2</sub> \*100 = 11 %*67 *\*\*\* Reproducibility at L<sub>2</sub> = 32 negative results divided by 36 samples \*100 = 88.9 %*

68

69 **Table XI. Global analysis of the results of all the participants in term of**  
 70 **repeatability of the analyses with the Premi®Test.**

Lab	(Number of identical results for the same sample / N)*100	(Number of identical results for 2 identical samples (pair) / N)*100
A	87.5	87.5
B	100.0	100.0
C	100.0	93.8
D	93.8	100.0
E	71.9	96.9
F	96.9	78.1
G	96.9	90.6
M	90.6	90.6
N	96.9	96.9
<b>Total</b>	<b>94.8</b>	<b>92.7</b>

73 *N: total number of samples (32)*

74

75