

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

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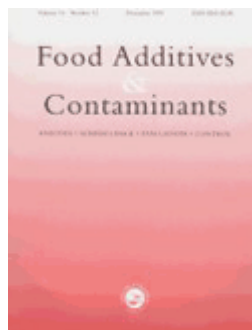
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AFNOR validation of PremiTest, a microbiological-based screening tube test for the detection of antimicrobial residues, in muscles from different animal origins

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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**

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5
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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)).

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.

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.

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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 3rd
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 $\mu\text{g kg}^{-1}$).

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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

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

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3 290 the number of negative results obtained for contaminated samples (spiked samples) divided
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5 291 by the total number of negative samples (the same number plus the number of blank
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7 292 samples giving a negative result) and multiplied by 100. The false negative rate calculated at
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9 293 1*MRL was equal to 22 % (= 10/(10+34)). However, the false negative rate calculated at
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11 294 twice the MRL was equal to 8 % (= 3/(3+34)).
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16 296 *Step 2. Treatment of animals and analysis of incurred porcine muscle samples.* The
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18 297 concentrations of naturally incurred samples were much higher than the respective MRL of
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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).

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3 318 The relative accuracy, the relative specificity and the relative sensitivity were calculated as
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5 319 70%, 42.9% and 84.6% respectively. Therefore, the relative accuracy and specificity were
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7 320 quite satisfactory. The low value of relative specificity could be explained because the
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9 321 sensitivity of the FPT was sometimes insufficient for certain antibiotics.
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14 323 In conclusion, FPT and Premi®Test gave concordant results when looking at the
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16 324 performance characteristics (relative accuracy, the relative specificity and the relative
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18 325 sensitivity). However, at this step, the false negative and the false positive rates were lower
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20 326 for the Premi®Test (both 0 %) than for the FPT (33 and 40 % respectively).
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25 328 *Step 3. Analysis of field samples.* 1427 field incurred samples, originated from 6 French field
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27 329 veterinary laboratories, were analyzed implementing Premi®Test and *Bacillus cereus* plate.
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29 330 1325 samples were detected negative and 102 samples doubtful (36) and positive (66) with
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31 331 the Premi®Test.
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35 333 Furthermore, among the 1325 negative results, 10 samples were detected positive with the
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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
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51 341 Premi®Test and positive with *Bacillus cereus* in the field laboratories, 6 were found positive
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53 342 or doubtful with Premi®Test at the CRL and confirmed positive with *Bacillus cereus*. The 4
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55 343 other samples were found positive only on plate Bc6. Finally, the presence of a tetracycline
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57 344 was confirmed by LC/MS-MS in 9 of these samples. Therefore, *Bacillus cereus* plate (one
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3 345 plate of the STAR protocol, which is selective for the detection of tetracyclines) is more
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5 346 sensitive for tetracyclines than Premi®Test.
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7

8 347 “[Insert Table IV about here]”
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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

24 355
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
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33 360 samples).
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36 361
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.
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42 364 “[Insert Table V about here]”
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47 366 When the data analysis was based only on the 112 samples reanalysed in the expert
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49 367 laboratory, relative accuracy (33.9%) and relative specificity (33.4%) were very low. Only
50
51 368 relative sensitivity (70 %) was satisfactory, as in the first step of the validation study.
52
53 369 However, in this case, Premi®Test negative samples (1315 negative samples / 1427
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55 370 analyzed samples) obtained in the field laboratories were not taken into account. Only the
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57 371 positive samples in the field laboratories were reanalysed at the CRL with Premi®Test and
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59 372 FPT. None of the 1315 negative samples was analysed with the FPT. By experience (a
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
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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
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11 405 same number plus the number of contaminated samples showing positive results) and
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13 406 multiplied by 100.
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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.
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36 417 After a positive result with Premi®Test, the presence of antimicrobial residues was
37
38 418 confirmed in 26 samples (33 %), from which 4 samples contained antimicrobials at
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40 419 concentrations upper than the MRL. Three of the 10 positive FPT samples and 4 of the
41
42 420 STAR positive samples were at concentrations upper than the MRL. Premi®Test and STAR
43
44 421 screened positive 22 and 13 samples respectively which really contained antimicrobial
45
46 422 compounds at concentrations lower than MRL. The FPT detected only 6 of these samples.
47
48 423 Therefore, the sensitivity of Premi®Test and STAR protocol was lower than those of the FPT.
49
50 424 The highest confirmatory rate was obtained for the FPT (90 %). The false negative rates of
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52 425 the FPT, the Premi®Test and the STAR protocol were equal to 8 %, 28 % and 20 %
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54 426 respectively. The false negative rate was calculated as the number of false negative results
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56 427 divided by the total of the true negative samples plus the number of false negative results for
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58 428 each method.
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3 429 The most commonly detected antimicrobial classes detected during this pilot study were
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5 430 beta-lactams (penicillin and amoxicillin) (15 samples) and tetracyclines (doxycycline,
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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
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33 444 samples really containing antimicrobial residues) with the pilot study.
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“[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.

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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
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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
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11 459 lower for sulphonamides (4 positive samples Premi®Test, 4 negative samples FPT). Two of
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13 460 these samples really contained sulphonamides at concentrations higher than the MRL and
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15 461 the FPT failed to detect them while the Premi®Test succeeded to detect. However, the
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17 462 sensitivity of FPT for tetracyclines was better (15 doubtful or positive samples with
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19 463 Premi®Test instead of 19 positive samples with FPT).
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27 465 The most detected antimicrobials were tetracyclines (25 samples) and beta-lactams (17
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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
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43 474 because the L3 concentration was that designed to give positive results, whereas the L2
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45 475 concentration was to be at the limit of sensitivity of the test.
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51 477 It should be noted that the later analysis of the results was carried out while taking as
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53 478 principle that the doubtful results are positive results, this which is applied at the time for
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55 479 routine analyses.
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57 480 “[Insert Table VIII about here]”
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3 482 The results of the expert laboratory were very satisfactory (Table VIII): 14 blank samples out
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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).
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16 488 *Results of the participants.* Before the study, the expert laboratory stated that the results of
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18 489 one laboratory would be removed when: the negative control was detected positive, the
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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,
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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.
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37 498 “[Insert Table IX about here]”
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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
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46 503 or 2 samples doubtful or positive, sometimes in only one series of analyses (laboratories M
47
48 504 and N).
49
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51 505 “[Insert Table X about here]”
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57 507 For the samples containing antibiotics at a concentration below the detection limit (L1), 6
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59 508 laboratories found 100% of negative results; 3 laboratories found 1 or 2 samples which were
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509 doubtful or positive. When the concentration was considered near the detection limits (L2),

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3 510 tylosin was generally detected (83 % of positive results), whereas the other antibiotics were
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5 511 less detected (oxytetracycline 11%, ceftiofur 11% and sulfamethazine 17%).
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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
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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.
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25 520
26 521 Table X presents also the analysis of the results of all the participating laboratories in term of
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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
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34 525 contaminated with an antimicrobial concentration exceeding the detection limit) on the total
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36 526 number of analyses.
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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.
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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

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2
3 566 method for antibiotic residues. On the contrary, the false positive rate of Premi®Test
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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
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11 570 detected because Premi®Test was more sensitive than FPT for some antimicrobials (beta-
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13 571 lactams and sulphonamides). Beta-lactams and some sulphonamides were satisfactorily
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15 572 detected at the MRL level by the Premi®Test.
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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
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33 580 of repeatability and reproducibility are very satisfactory, with average percentages of 94,8%
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35 581 and 92,7 % for the repeatability and of 89,1 for reproducibility.
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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
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49 587 using as negative control a "blank" muscle of each analyzed specie to optimise the reading
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51 588 time.
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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.

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601 study.

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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
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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

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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	Global
MRL(muscle) ($\mu\text{g kg}^{-1}$)	100	100	100	50	50	1000	/
Tested concentrations ($\mu\text{g kg}^{-1}$)	50/ 100 /200	50/ 100 /200	50/ 100 /200	25/ 50 /100	50 /100/200	100/200/400	/
Detection rate at 0.5 MRL	0%	60%	80%	100%	(0%)*	-	48%
Detection rate at MRL	20%	80%	100%	100%	0%	(100%)	67%
Detection rate at 2* MRL	100%	100%	100%	100%	40%	(100%)	90%
Detection capability	2xMRL	2xMRL	MRL	0.5xMRL	> 2xMRL	0.5xMRL	/

7 **MRL in bold character*

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

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

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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 [®] 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
LVD	Number of samples analysed at LVD ^b	379	671	205	26	146	1427
	Number of positive and doubtful results at LVD Premi®Test	63	26	1	5	7	102
AFSSA	Number of samples analysed at AFSSA	65	31	4	5	7	112^a
	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
AFSSA LC/MS-MS	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 ^a Total of the samples sent to AFSSA for confirmation

21 ^b 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 nd 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	
Global	<LMR	26	4	7	15	20	6	13	13
	>LMR	5	1	0	4	2	3	1	4
	Total	31	5	7	19	22	9	14	17
Absence	/	57	5	8	44	56	1	43	14
Not analysed	/	24	24			24		24	
TOTAL	/	112	34	15	63	102	10	81	31

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
	Sum***	17					
Tetracyclines	<MRL	13	5	4	4	6	8
	>MRL	12	5	1	6	/	11
	Sum	25					
Sulphonamides	<MRL	2	/	1	1	2	/
	>MRL	2	/	/	2	2	/
	Sum	4					
Quinolones	>MRL	3	1	/	2	/	3
BL + Sulfa	<MRL	2	/	/	2	1	1
	>MRL	2	/	/	2	2	/
	Sum	4					
Tetra + Sulfa	<MRL	1	/	/	1	/	1
	>MRL	3	/	/	3	/	3
	Sum	4					
Tetra + BL	<MRL	1	/	/	1	1	/
Macrolides	<MRL	3	1	1	1	1	2
	>MRL	3	/	/	3	1	2
	Sum	6					
Global	<MRL	31	6	7	18	19	13
	>MRL	33	6	1	26	7	25
TOTAL	/	64	12	8	44	26	38

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⁻¹).

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	L0	FP0 a)*	0%	100.0
	2	L1	FP1 b)	0%	100.0
	3	L2	TP2 c)	11%**	88.9***
	4	L3	TP3 d)	69%	69.4
Porcine ceftiofur	5	L0	FP0 a)	11%	83.3
	6	L1	FP1 b)	8%	94.4
	7	L2	TP2 c)	17%	88.9
	8	L3	TP3 d)	53%	100.0
Bovine sulfamethazine	9	L0	FP0 a)	17%	91.7
	10	L1	FP1 b)	6%	91.7
	11	L2	TP2 c)	11%	83.3
	12	L3	TP3 d)	100%	52.8
Chicken Tylosin	13	L0	FP0 a)	0%	100.0
	14	L1	FP1 b)	0%	100.0
	15	L2	TP2 c)	83%	83.3
	16	L3	TP3 d)	100%	100.0
Total					89.1

57

58

59 *FP : False positive rate*60 *TP : True positive rate*61 *a) False positive at level L₀*62 *b) False positive at level L₁*63 *c) True positive at level L₂*64 *d) True positive at level L₃*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 L₂ *100 = 11 %*67 **** Reproducibility at L₂ = 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
Total	94.8	92.7

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

74

75