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

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

4  
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1  
2 19 **Abstract**

3  
4  
5 20 EXPLORER® is a simple and fast new kit for detection of inhibitory substances in raw meat. The  
6  
7 21 test, a 96-well microtiter plate, is based on the inhibition of microbial growth (*Geobacillus*  
8  
9 22 *stearothermophilus* spores). The Explorer® test was validated in accordance with the decision  
10  
11 23 2002/657/EC (EC 2002). The specificity and detection capabilities for 5 compounds from major  
12  
13 24 antimicrobial families and robustness were studied. The specificity of the test was assessed with 4  
14  
15 25 different animal species and was found to be very satisfactory (false positive rates lower than 10  
16  
17 26 %). The detection capabilities for amoxicillin (10 µg kg<sup>-1</sup>) and tylosin (100 µg kg<sup>-1</sup>) were at the MRL  
18  
19 27 level (50 and 100 µg kg<sup>-1</sup> respectively) for both, for doxycycline (200 µg kg<sup>-1</sup>) and sulfathiazole (200  
20  
21 28 µg kg<sup>-1</sup>) at 2 times the MRL (100 µg kg<sup>-1</sup> for each) and for cefalexin (500 µg kg<sup>-1</sup>) at 2.5 times the  
22  
23 29 MRL (200 µg kg<sup>-1</sup>). Twenty-one samples were analysed in parallel with the Four Plate Test, the  
24  
25 30 STAR protocol and the Explorer® test. One false positive result and 2 false negative results  
26  
27 31 (samples containing oxytetracycline) were reported with the Explorer® test. In conclusion, the  
28  
29 32 Explorer® test was shown to be robust and easily automated. Photometric reading allows  
30  
31 33 informatic data storage and objective readings between technicians and days. The Explorer® test  
32  
33 34 could be used as a wide screening test because it enables detection of most of the antimicrobial  
34  
35 35 families (penicillins, cephalosporins, tetracyclines, sulphonamides and macrolides) in muscles from  
36  
37 36 different animal species (porcine, bovine, ovine, poultry).  
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43

44 38 **Keywords:** *Validation, Explorer, inhibition test, antimicrobial screening assay, muscle samples,*  
45  
46 39 *animal species, photometric reading*  
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## 1 2 40 **Introduction**

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4  
5 41 The presence of antimicrobial residues in food of animal origin could be due to treatment of animal  
6  
7 42 disease, or prophylaxis purposes. The risks for consumer health due to the presence of  
8  
9 43 antimicrobial residues could be: toxicological effects, allergies (especially with beta-lactam  
10  
11 44 residues), or antimicrobial resistance of pathogenic bacteria. Therefore, Maximum Residue Limits  
12  
13 45 (MRLs) have been set for antimicrobial residues in the European Union (2377/90/EC) (EC 1990).  
14  
15 46 There are a large number and the variety of antimicrobial families. Moreover, the antibiotics used in  
16  
17 47 different countries are generally different. Therefore, the tests which are commercialised all over  
18  
19 48 the world have to be able to detect a maximum number of these antibiotics having an MRL. More  
20  
21 49 than sixty different antimicrobials have MRLs in animal tissues. So the detection of antimicrobial  
22  
23 50 residues should be performed with wide screening tests, which are the first level of official control,  
24  
25  
26 51 at MRL level.  
27

28 52  
29  
30 53 Different methods have already been developed and proposed for screening of antimicrobials in  
31  
32 54 muscle. Different microbiological plate tests are available: Four Plate Test (FPT) (Bogaerts et al.  
33  
34 55 1980), a Five Plate Test developed at the Community Reference Laboratory (CRL) called the  
35  
36 56 STAR protocol (Screening Test for Antibiotic Residues) (Gaudin et al. 2004), other plate tests: a  
37  
38 57 one-plate method (Koenen-Dierick et al. 1995), a modified EC Four Plate Method to detect  
39  
40 58 antimicrobial drugs (Okerman et al. 1998, Currie et al. 1998), six-plate tests for meat samples  
41  
42 59 (Myllyniemi et al. 2001, Ferrini et al. 2006). The European Four Plate test is commonly used for  
43  
44 60 detection of antibiotics in food. This method requires stabilisation of several bacteria strains and  
45  
46 61 takes about 24 hours to produce results. Moreover, the detection capability of this assay is well  
47  
48 62 above the MRL for sulphonamides.  
49

50 63  
51  
52 64 More recently, some tube tests have been developed for the screening of antimicrobial residues in  
53  
54 65 meat as proposed for milk analysis. Generally, these tube tests are based on the strain  
55  
56 66 *Geobacillus stearothermophilus* (formerly *Bacillus stearothermophilus*). The ampoule contains a  
57  
58 67 pre-seeded medium, with a pH change indicator. The reading is based on the change of colour of  
59  
60 68 the medium because of a redox indicator which produces a colour change in the test when

1  
2 69 antibiotics are not present in the sample (yellow for negative samples, purple for positive samples).  
3  
4 70 This kind of test is ready-to-use and easy-to-use, while plate tests are home-made methods and  
5  
6 71 need to maintain bacterial strain. The first commercial tube test for muscle was the Premi®Test  
7  
8 72 (DSM, Delft, The Netherlands). The incubation time of Premi®Test (2h30 to 2h45) is lower than for  
9  
10 73 plate tests (15 to 24 hours). More recently, a new test has been developed by another company  
11  
12 74 (Zeu-Inmunotec, Saragossa, Spain). This new test, called Explorer® test, is also based on the  
13  
14 75 bacterial strain *Geobacillus stearothermophilus*. The principle of the test is the same as  
15  
16 76 Premi®Test. However, Explorer® test uses a new microplate format (96 wells). The Explorer® test  
17  
18 77 has been optimized for different meat species: pork, chicken, beef, lamb, etc. by the manufacturer.  
19  
20 78 In our lab, Premi®Test was fully validated for its use on muscles from different animal species  
21  
22 79 (Gaudin et al. 2008)). The objective of the present study was to evaluate the performance  
23  
24 80 characteristics of the Explorer® kit from Zeu-Inmunotec (Saragossa, Spain) for the detection of  
25  
26 81 antibiotic residues in muscle from different animal species. To our knowledge, no studies of the  
27  
28 82 performance of this test have been published at this time. This article will present the results of a  
29  
30 83 preliminary validation study performed at the Community Reference Laboratory. The validation of  
31  
32 84 screening tests for the detection of antimicrobial residues was conducted as in the decision  
33  
34 85 EC/2002/657 (EC 2002). Detection capabilities (CC $\beta$ ), specificity, false positive rate were  
35  
36 86 calculated. Furthermore, a robustness study has been conducted on 4 parameters based on an  
37  
38 87 experimental plan.  
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## 46 89 **Materials and methods**

### 47 90 *Explorer® test*

48  
49 91 Explorer® test is a simple and fast kit for detection of inhibitory substances in raw meat, feed and  
50  
51 92 eggs. The test, a 96-well microtiter plate, is based on the inhibition of microbial growth. Each well  
52  
53 93 contains specific agar medium containing a redox indicator and spread with *Geobacillus*  
54  
55 94 *stearothermophilus* spores.  
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1  
2 96 *Extraction of muscle samples for Explorer® test.* A piece of meat (2-3g) was cut and put into a  
3  
4 97 clean glass or heat resisting plastic tube. The tube was closed and heated it in a water bath at  
5  
6 98 100°C for 3 min. After the heating, some fluid was released from the sample. The meat sample  
7  
8  
9 99 was pressed with forceps to release as much fluid as possible. Finally the meat fluid was clarified  
10  
11 100 by centrifugation (2000 g for 3 min).

12  
13 101  
14  
15 102 *Test procedure.* 0.1 ml of meat fluid was added to each well with a micropipette. The wells were  
16  
17 103 sealed with an adhesive sheet and the microplate was pre-incubated at 65°C for 30 min in a block  
18  
19 104 heater (ref. ZE/FX – FX incubator supplied by Zeu-Inmunotec, Saragossa, Spain) to allow the  
20  
21 105 sample to diffuse through the well. Afterwards the wells were washed by filling the wells up with  
22  
23  
24 106 distilled water, using a squeeze bottle. The washing step was repeated 3 times. The plate was  
25  
26 107 emptied by turning the plate upside down on top of an absorbent paper to remove the water in  
27  
28 108 excess. The plate was sealed again with an adhesive sheet and incubated at 65°C until the  
29  
30 109 negative control sample has turned yellow (approximately 3h-3h30min).

31  
32 110  
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34  
35 111 *Quality controls (QC)*

36  
37 112 It was strongly recommended by the manufacturer to use a negative control sample (antibiotic-free  
38  
39 113 meat) to determine the optimal incubation time of each assay. It was also advisable to include a  
40  
41  
42 114 positive sample (meat sample with high concentration of antibiotic, i.e. Penicillin G 25 µgkg<sup>-1</sup> in  
43  
44 115 juice extract). Penicillin G was supplied by Sigma-Aldrich (Saint-Quentin-Fallavier, France).

45  
46 116 2 different negative controls were used:

- 47  
48 117 - controls prepared with meat from the same animal species as the meat tested (prepared by  
49  
50 118 ourselves),  
51  
52 119 - a control supplied by Zeu-Inmunotec (Saragossa, Spain) which was a mix of juices from  
53  
54 120 different animal species (lyophilised form to be reconstituted).

55  
56  
57 121  
58  
59 122 *Readings and interpretation of Explorer results.* When the plate was incubated at 65°C, spores  
60  
123 germinate and cells grow modifying the redox potential of the medium The agar colour changed

1  
2 124 from blue to yellow if the sample did not contain antibiotics or antimicrobial residues at  
3  
4 125 concentrations below the detection limits of the test. If samples contained antibiotics at  
5  
6 126 concentrations above the detection limit of the kit, microorganisms will not grow neither colour  
7  
8 127 changes will be observed.  
9

10  
11 128  
12  
13 129 The photometric reading of microplates is based on the reading at 2 wavelengths (590 and 650  
14  
15 130 nm) on a microplate reader. The results were interpreted as the difference between the values of  
16  
17 131 the two readings.  
18

19 132 An example of interpretation of quantitative results (Optical Density) is presented on Figure 1.  
20

21 133 “[Insert Figure 1 about here]”  
22

23  
24 134 At each day of validation, one negative control of each species was analysed and also one positive  
25  
26 135 control (penicillin G at 25  $\mu\text{gkg}^{-1}$ ). Then the results of each unknown sample from each species  
27  
28 136 were compared to the corresponding negative control. The plate is ready to be read when the  
29  
30 137 result for the negative control sample (difference of absorbance NA590nm - NA650 nm) between  
31  
32 138 0.15 and 0.25 OD (optical density units), where NA: Negative control absorbance. A sample is  
33  
34 139 declared positive when: SA 590nm - SA 650 nm  $\geq$  NA590nm - NA 650nm + 0.15, where : SA:  
35  
36 140 Sample absorbance.  
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39 141  
40

#### 41 142 *Validation*

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43  
44 143 *Specificity and false positive rate.* 20 blank meat samples from different origins should be analysed  
45  
46 144 in blind duplicate with Explorer® kit. The applicability of the test to meat from different animal  
47  
48 145 species was studied simultaneously with specificity determination because the 20 meat samples  
49  
50 146 were from different species: 5 porcine meat, 5 bovine meat, 5 poultry meat and 5 ovine meat.  
51

52 147  
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54 148 *Detection capabilities* Five different antimicrobials were tested at the MRL and at the announced  
55  
56 149 detection limit (manufacturer data): amoxicillin (penicillin family), cefalexin (cephalosporin family),  
57  
58 150 doxycycline (tetracycline family), sulfathiazole (sulphonamide family) and tylosin (macrolide family).  
59  
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1  
2 151 Antibiotic and sulphonamide standards were provided by Sigma Aldrich (Saint-Quentin Fallavier,  
3  
4 152 France).

5  
6 153 First the fluid from blank muscle samples was extracted by pressing the meat with a garlic press.  
7  
8 154 Then the juice samples were spiked with antibiotic solutions. Therefore, the validated  
9  
10 155 concentrations are the real concentrations in the juice samples. Twenty samples (5 porcine, 5  
11 156 bovine, 5 ovine and 5 poultry) fortified at 2 levels with each antimicrobial were prepared “blind to  
12  
13 157 the analyst” and analysed in duplicate on 4 different days (5 samples per day). Blank samples and  
14  
15 158 spiked samples were aliquoted and stored frozen over 1 week maximum to prevent stability  
16  
17 159 problems with antimicrobials. The blank samples could be analysed during a longer period  
18  
19 160 because there is no problem of stability.  
20  
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24 161  
25  
26 162 At the end of the validation, 5 spiked samples for each antibiotic at each concentration and 5 blank  
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28 163 samples, from each species, have been analysed. In the case of substances with an established  
29  
30 164 MRL, the detection capability is the concentration at which the method is able to detect a  
31  
32 165 compound at the MRL with a statistical certainty of  $1 - \beta$ . The detection capability  $CC\beta$  was  
33  
34 166 calculated as the concentration where less than 5% of false compliant results remains. When one  
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36 167 sample or less among 20 samples spiked at the MRL is detected negative, the detection capability  
37  
38 168 was equal to or lower than the MRL.  
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41 169  
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43 170 *Robustness study.* Robustness studies use the deliberate introduction of minor reasonable  
44  
45 171 variations by the laboratory and the observation of their consequences. Factors which may  
46  
47 172 influence the measurement results have been selected: sample volume, pre-incubation time, pre-  
48  
49 173 incubation temperature and incubation temperature. These factors should be modified in an order  
50  
51 174 of magnitude that matches the deviations usually. The robustness study has been focused on one  
52  
53 175 representative antimicrobial amoxicillin and on porcine muscle. 10 blank samples and 10 different  
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55 176 samples, spiked at the detection capability of amoxicillin were blindly analysed, in duplicate, at  
56  
57 177 different days.  
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178

1  
2 179 An experimental plan which combined the different factors was built to minimise the experiments.  
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4 180 A full factorial design was chosen. Based design was 2-level factorial design for 3 factors (volume  
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6 181 of samples, pre-incubation time and pre-incubation temperature), noted by letters A, B and C. For  
7  
8 182 each of these factors, a low value (“-“) and a high value (“+“) (low and high compared to normal  
9  
10 183 conditions) have been tested. Considering the 3 factors and 2 levels for each one (“-“, “+“), the  
11  
12 184 total number of experiments was 8 (8 different days). On each different day, 10 blank samples and  
13  
14 185 10 spiked samples have been analysed. Experimental runs were coded as ‘-‘ for low levels and ‘+’  
15  
16 186 for high levels (Table I).

17  
18  
19 187 “[Insert Table I about here]”  
20

21 188 A fourth factor (incubation temperature) has been studied by adding negligible interaction to the  
22  
23 189 experimental design (Renard et al. 1992). Incubation temperature (letter D=ABC) was studied at  
24  
25 190 levels defined by interaction between the 3 factors of the base design (ABC). “D” and “ABC” were  
26  
27 191 alias structure. The design matrix is presented in Table II.

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29  
30 192 “[Insert Table II about here]”  
31

32 193 Therefore, 4 factors have been studied (8 combinations of experiments = 8 runs = 8 days). The  
33  
34 194 influence of these factors on false positive and false negative rates and on incubation time as well  
35  
36 195 as the interactions between factors were evaluated. For each run (day), the response is indicated  
37  
38 196 in Table III (false positive rate, false negative rate and on incubation time).

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41 197  
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43 198 *Comparison with other screening methods and confirmation of positive samples by LC/MS-MS*  
44  
45 199 *(Incurred tissues)*. The Four Plate Test (FPT) is the French official method for the screening of  
46  
47 200 antimicrobials in muscle (Bogaerts et al. 1980). The STAR protocol is a Five Plate Test which was  
48  
49 201 developed and validated for the screening of antimicrobial residues in milk, by the Community  
50  
51 202 Reference Laboratory for antimicrobial residues (AFSSA Fougères) (Gaudin et al. 2004). The  
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53 203 STAR protocol is also applicable to the screening of antimicrobial residues in muscle and is in  
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55 204 progress to be validated for muscle screening. The 2 plate tests are able to detect a wide range of  
56  
57 205 antibiotics (beta-lactams, tetracyclines, macrolides, quinolones, aminoglycosides, sulfonamides).  
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59 206 The detection limits of these tests are very different between families of antibiotics (beta-lactams  
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207 and tetracyclines are the best detected) and between antibiotics inside each family. Moreover, the

1  
2 208 detection limits of the STAR protocol for most of the antibiotics are lower than detection limits of  
3  
4 209 the FPT. It is particularly obvious with sulfonamides and quinolones. Twenty-one muscle samples  
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6 210 from different animal species were collected from the field laboratories in France and were  
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8  
9 211 analysed in AFSSA Fougères in parallel with the Explorer® kit, the FPT and the STAR test.  
10  
11 212 Finally, all the positive samples with at least the FPT or the STAR protocol were confirmed by a  
12  
13 213 multi-residue LC/MS-MS method (Gaudin et al. 2008). The principle is based on two different  
14  
15 214 extractions : one with trichloroacetic acid (TCA) allowing the detection of tetracyclines,  
16  
17 215 aminoglycosides and quinolones, the second with acetonitrile (ACN) allowing the detection of  
18  
19 216 penicillins, cephalosporins, macrolides and sulphonamides. LC/MS-MS is used with a Multi  
20  
21 217 Reaction Monitoring Mode (MRM) and two MRM transitions are monitored for each compound.  
22  
23  
24 218 Fifty monitored compounds excepted some aminoglycosides were detected in muscle at a level  
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26 219 below the MRL.

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## Results and discussion

### *Specificity and false positive rate*

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

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

1  
2 236 lower than 5%. Consumer safety is more important than the cost of analyses, even if the  
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4 237 developer of screening methods have to be informed of the consequences of high false positive  
5  
6 238 rates.  
7

8 239  
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10  
11 240 To increase the level of confidence in the determination of the false positive rate, more than 20  
12  
13 241 blank samples would have to be tested. This first determination gave us an estimation of the false  
14  
15 242 positive rate. More experiments would have to be performed in the future to complete the  
16  
17 243 validation.  
18

### 19 244 20 21 245 *Detection capabilities*

22  
23  
24 246 The detection capabilities were calculated for the 5 tested antimicrobials and compared with those  
25  
26 247 indicated by the manufacturer (Table III).  
27

28 248 “[Insert Table III about here]”  
29

30  
31 249 The Maximum Residue Limit (MRLs) for amoxicillin, doxycycline, sulfathiazole and tylosin are  
32  
33 250 respectively 50, 100, 100 and 100  $\mu\text{g kg}^{-1}$ . The  $\text{CC}\beta$  values of amoxicillin and tylosin were at the  
34  
35 251 MRL level. The detection capabilities of doxycycline and sulfathiazole are at 2 times the MRL level  
36  
37 252 and around 3 times the MRL level for cefalexin. The detection capabilities determined during this  
38  
39 253 validation were similar to those claimed by the manufacturer. Two different negative controls have  
40  
41 254 been tested and used to calculate the cut-off value to classify unknown samples as negative or  
42  
43  
44 255 positive. The first control fluid was prepared with meat from the same animal species as the meat  
45  
46 256 tested (prepared by ourselves). The second control was supplied by Zeu-Inmunotec (Saragossa,  
47  
48 257 Spain) and was a mix of juices from different animal species. The choice of the negative control  
49  
50 258 from Zeu-Inmunotec (Saragossa, Spain) could be interesting regarding the following results. There  
51  
52 259 were no influence of the negative control on the  $\text{CC}\beta$  values of amoxicillin, tylosin, doxycycline and  
53  
54  
55 260 sulfathiazole. There was only a slight influence on the detection of cefalexin. The  $\text{CC}\beta$  was a little  
56  
57 261 lower when the negative control was that from Zeu-Inmunotec (Saragossa, Spain). Moreover, it  
58  
59 262 would be easier to use a prepared negative control like it (lyophilised), instead of preparing a  
60  
263 negative control, from each species, in the laboratory implementing the kit. Therefore, the use of

1  
2 264 this negative control could be recommended for practical, technical and economical reasons, even  
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4 265 if the false positive rate seems to be higher (10 % instead of 5 %).  
5

6 266  
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8  
9 267 The detection capabilities were calculated after the analyses of muscles from 4 different species.  
10  
11 268 No influence of the animal species was observed on the  $CC\beta$  values. The same conclusion was  
12  
13 269 reported with the Premi®Test by Cantwell et al. (2006).  
14

15 270  
16

### 17 271 *Robustness study*

18  
19  
20 272 The influence of 4 factors (volume of samples, pre-incubation time, pre-incubation temperature  
21  
22 273 and incubation temperature) on false positive and false negative rates and on incubation time as  
23  
24 274 well as the interactions between factors were evaluated. For each run (day), the response is  
25  
26  
27 275 indicated in Table III (false positive rate, false negative rate and on incubation time). These values  
28  
29 276 were added together. Than the result was divided by the number of runs (8 in this case). A  
30  
31 277 quantitative value of the effect of each factor in relation to average of runs (column noted I was  
32  
33 278 then obtained (Table IV).

34  
35 279 “[Insert Table IV about here]”  
36

37 280 False positive rate. The false positive rate did not change with sample volume (A), pre-  
38  
39  
40 281 incubation time (B) and pre-incubation temperature (C). A negative effect of interaction  $D=ABC$   
41  
42 282 was observed on false positive rate, that means that false positive rate decreased when factor D  
43  
44 283 (incubation temperature) increased. On the contrary, a positive effect of interaction  $AB+CD$  and  
45  
46 284  $AC+BD$  was observed. The false rate positive rate increased when these factors interacted.  
47

48 285 False negative rate. The false negative rate decreased with sample volume (A) and pre-  
49  
50  
51 286 incubation time (B) and with the interaction  $BC+AD$ , that means that the false negative rate  
52  
53 287 decreased when sample volume and pre-incubation time increased. This conclusion was not  
54  
55 288 surprising because it is well known that for these kind of tests, the detection capability of the test is  
56  
57 289 better if the sample volume is higher and pre-incubation time longer. A negative effect on false  
58  
59 290 negative rate was observed with interaction  $BC+AD$ . Therefore, the interaction of these factors  
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291 reduced the false negative rate. However, a positive effect of  $D=ABC$  (incubation temperature) and

1  
2 292 interactions AB+CD and AC+BD. Therefore, the false negative rate increased when incubation  
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4 293 temperature increased. The most important effect was observed on run 5 where volume of  
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6 294 samples and pre-incubation time were lowered and pre-incubation temperature and incubation  
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8 295 temperature were increased. The false negative rate was equal to 27.3 %. Pre-incubation  
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10 296 temperature (C) had no effect on the false negative rate. In the protocol of Explorer® test, there is  
11  
12 297 no recommended standard deviation on pre-incubation and incubation temperature at 65°C. This  
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14 298 study proved that pre-incubation temperature could vary from 62 to 68°C without influence on the  
15  
16 299 detection capability of amoxicillin. However, the false negative rate increased when incubation  
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18 300 temperature increased.

21  
22 301 Incubation time. Factor B (pre-incubation time) had the most important effect on the  
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24 302 incubation time. When pre-incubation time decreased, incubation time increased and conversely. It  
25  
26 303 is well known that for these kind of tests, the detection capability of this kind of test decreased  
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28 304 when incubation time is too long. However, as the end-point of the test is based on the optical  
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30 305 density of the negative control and not on a finite time, this consideration does not affect the  
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32 306 ruggedness of the assay.

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37 308 As a conclusion, individual factors (except factor D) had few or not at all impact on the detection  
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39 309 capability or specificity of the test. Factor D (incubation temperature) increased the false negative  
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41 310 rate significantly. When incubation temperature increased, false negative rate increased and false  
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43 311 positive rate decreased. Factor D The incubation temperature was the most critical factor. Finally,  
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45 312 the interactions of factors AB+CD and AC+BD had a bad effect on the performance of the test  
46  
47 313 because false positive and false negative rates increased.

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52 315 The requirements regarding a screening test for veterinary drugs are a false negative rate equal or  
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54 316 lower than 5% and a false positive rate as low as possible. Therefore, the Explorer® test was  
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56 317 found to be rugged with respect to animal species, sample volume, pre-incubation time, pre-  
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58 318 incubation temperature and incubation temperature.

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1  
2 320 *Comparison with other screening methods and confirmation of positive samples by LC/MS-MS*  
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4 321 *(Incurred tissues)*. Twenty-one incurred muscle samples from the field laboratories were analysed  
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6 322 with 3 microbiological methods. Different animal species were analysed: 13 bovine, 6 porcine, 1  
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9 323 turkey, 1 unknown. These samples were taken randomly in the pool of samples arriving in our  
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11 324 laboratory. The results of all analyses are presented in Table V. At least 7 samples contained very  
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13 325 high concentrations of antimicrobials. Therefore, these samples were easily detected by the 3  
14  
15 326 microbiological tests (Explorer® kit, Four Plate test and STAR test).

16  
17 327 “[Insert Table V about here]”  
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19 328 The samples which were detected positive with one screening test, but which finally did not contain  
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22 329 antimicrobial residues or residues at concentrations lower than MRL (after the LC/MS-MS  
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24 330 confirmation) were considered as false positive results. Seven false positive results were reported  
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26 331 with the STAR protocol and 4 with the FPT. For 3 of them (2 porcine and 1 bovine samples), the  
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28 332 explanation was that these 2 methods were able to detect tetracyclines, even at concentrations  
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30 333 lower than MRL (too sensitive).  
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35 335 Concerning the 4 other false positive results obtained with the STAR protocol and the one  
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37 336 remaining false positive result of the FPT (1 bovine sample), either it was real false positive results  
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39 337 (presence of natural inhibitors) or the antimicrobial residue was one of those which was not  
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41 338 detected by the multi-residue LC/MS-MS method. The good example is sample codified v-1510  
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43 339 which was positive with both FPT and STAR protocol, but confirmed negative by LC/MS-MS. Only  
44  
45 340 1 false positive result was obtained with the Explorer® test (bovine sample). However, because  
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47  
48 341 this sample was negative with the STAR protocol and the FPT (official method), it was not  
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50 342 confirmed by LC/MS-MS. Therefore, it was impossible to be sure that it was a real false positive  
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52 343 result.  
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56 345 Only the samples really containing antimicrobials at concentrations upper than MRL and not  
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59 346 detected by the screening test were considered as false negative results. The STAR protocol did  
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347 not give any false negative results (0%). The Four Plate Test (FPT) gave 1 false negative result  
348 (4.7%) and the Explorer® test 2 false negative results (9.5%). The FPT failed to detect amoxicillin

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2 349 in a bovine sample, at a concentration higher than the MRL. The Explorer® test did not detect  
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4 350 oxytetracycline in 2 different samples: a turkey sample at 140 µg kg<sup>-1</sup> and a bovine sample at 315  
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6 351 µg kg<sup>-1</sup>. This was not surprising because with most of the microbiological tests, tetracyclines are  
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9 352 not generally detected at the MRL level (100 µg kg<sup>-1</sup>), especially when only one bacterial strain was  
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11 353 used for detection (i.e. Premi®Test) (Gaudin et al. 2008), but a little higher. Furthermore, when the  
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13 354 incubation increased, the detection capability decreased rapidly, especially for tetracyclines.  
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15 355 Okerman et al. (2004) also reported a poor detection capability of Premi®Test towards  
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17 356 tetracyclines in muscle tissue. At the MRL level, no positive results over 3 tests were reported for  
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19 357 the 4 tetracyclines (tetracycline, oxytetracycline, chlortetracycline and doxycycline). Moreover,  
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21 358 incurred chicken tissue with doxycycline at 108 µg kg<sup>-1</sup> was found negative also. Premi®Test was  
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23 359 sensitive enough for sulphonamides and some macrolides. The Explorer® test was also found  
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25 360 sensitive enough for 2 antibiotics of these families (sulfathiazol and tylosin).  
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- 28  
29 361 - Two points should be underlined concerning the filed samples: firstly, some antibiotics were  
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31 362 present at very high concentrations, much higher than MRLs.  
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- 33 363 - Secondly, 9 samples out of 21 contained several antibiotics from the same family and/or  
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35 364 from different families.  
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37 365 The high concentrations contained in real samples sometimes could be due to the fact that the  
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39 366 sample has been taken at injection point. Moreover, samples with the concentrations nearest from  
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41 367 the respective MRL of antimicrobials should be more interesting to conclude on the performance of  
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43 368 the tests. However, field samples were randomly taken and before confirmatory analyses, we had  
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45 369 no idea of the antimicrobial concentration. For this reason, a parallel validation with spiked juice  
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47 370 samples at the target concentration (MRL) was really necessary to determine detection capabilities  
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49 371 of the Explorer® test. To our knowledge, there were no scientific publications at this time  
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51 372 presenting results obtained with the Explorer® test. However, there were some validation studies  
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53 373 performed on the Premi®Test. In the classical protocol of Premi®Test, there is no solvent  
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55 374 extraction of muscle samples, only fluid extraction with a garlic press. Stead et al. (2004) proposed  
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57 375 an extraction protocol (solvent extraction) before using the Premi®Test. The validation study  
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59 376 compared the detection limits of the 2 different protocols. The detection limits were generally lower  
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1  
2 377 with the solvent extraction than with the fluid extraction. The detection limits with a fluid extraction  
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4 378 only for amoxicillin, doxycycline, sulfathiazole and tylosin were respectively > 5, 50, 25 and <25  $\mu\text{g}$   
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6 379  $\text{kg}^{-1}$ . Here, the detection capabilities of the Explorer® Test for amoxicillin, doxycycline, sulfathiazole  
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8 380 and tylosin were respectively 10, 200, 200 and 100  $\mu\text{g kg}^{-1}$ . Therefore, the detection capability of  
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10 381 Premi®Test even with fluid extraction only, seemed to be better than the detection capability of the  
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12 382 Explorer® test. However, the detection limits and the  $\text{CC}\beta$  values were not determined in the same  
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15 383 way. Stead et al. (2004) determined the detection limits by analysing  $n=4$  spiked samples for each  
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17 384 analyte/matrix combination. The detection capability was determined for the Explorer® test after  
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19 385 the analyses of 20 samples and the  $\text{CC}\beta$  was calculated as the antibiotic concentration given less  
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22 386 than 5 % of false negative results. Therefore, the values were not directly comparable.  
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24 387 Furthermore, in our lab, the Premi®Test was validated for the detection of antimicrobial residues in  
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26 388 muscle (Gaudin et al. 2008)). The results were similar to those obtained with the Explorer® test  
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29 389 during our validation study for the 2 common tested antibiotics, amoxicillin ( $\text{CC}\beta \leq 50 \mu\text{g kg}^{-1}$ ) and  
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31 390 tylosin ( $\text{CC}\beta = 100\mu\text{g kg}^{-1}$ ).  
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33 391  
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35 392 The photometric reading of the Explorer® test was very interesting. It is well known that visual  
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37 393 evaluation of this kind of test is subjective. As it was observed with the Premi®Test (Stead et al.  
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39 394 2005), the use of a scanner technique removes the subjectivity of the analyst involved in visually  
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42 395 determining the end-point colour. The same conclusion could be underlined with the Explorer® test  
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44 396 and the photometric reading. The determination of a cut-off value from the quantitative value of the  
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47 397 negative control in the Explorer® protocol allows to classify negative and positive results from an  
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49 398 objective parameter.  
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## 51 399 52 53 400 **Conclusion**

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56 401 The Explorer® test (Zeu Inmunotech, (Saragossa, Spain) is applicable to the detection of different  
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58 402 antimicrobial families (penicillins, cephalosporins, tetracyclines, macrolides and sulfonamides) in  
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60 403 the muscles from different animal species (bovine, porcine, ovine and poultry). The detection  
404 capabilities of the 5 tested antimicrobials were at the MRL level or lower (amoxicillin, tylosin) and

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2 405 sometimes a little higher than the respective MRLs (sulfathiazole, doxycycline, cefalexin) (2 to 3  
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4 406 times the MRL). The applicability of Explorer® test to muscles from different animal species was  
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6 407 proved when a negative control from each species is tested for each assay. The sample  
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8 408 preparation is quick and simple. This test could be easily automated because of the microplate  
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10 409 format. High throughput of samples could be analysed. Furthermore, the advantages of  
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12 410 photometric reading were the objectivity of results avoiding variations due to visual reading made  
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14 411 by different technicians or performed over different days. Finally, spectrophotometric reading  
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16 412 allowed informatic registration, ensuring traceability of assays.  
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22 414 The results of Explorer® test for the analysis of incurred field samples were very satisfactory. This  
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24 415 demonstrates that this technique seems to be fit-for-purpose as a qualitative screening assay.  
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26 416 These results were very promising. However, some families of antimicrobials have not been tested  
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28 417 yet (e.g. aminoglycosides, quinolones). These families are generally not well detected by  
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30 418 microbiological tests, especially when all only bacterial strain is used. The next step should be to  
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32 419 validate the test for other families of antibiotics and a higher number of antimicrobials from the  
33  
34 420 main families.  
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37 421  
38

#### 39 422 **Acknowledgements**

40  
41 423 Thanks to Zeu-Inmunotec (Saragossa, Spain) for providing us with different batches of  
42  
43 424 EXPLORER® test.  
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2 470 **Figure 1. Example of raw data for day 1 of validation: Interpretation of the results.**

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4 471 At each day of validation, one negative control of each species was analysed and also one positive

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6 472 control (penicillin G at 25  $\mu\text{gkg}^{-1}$ ). Then the results of each unknown sample from each species

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8 473 were compared to the corresponding negative control. The plate was ready to be read when the

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10 474 result for the negative control sample (difference of absorbance NA590nm - NA650 nm) was

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12 475 between 0.15 and 0.25 OD (optical density units) where NA: Negative control absorbance. A

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14 476 sample is declared positive when: SA 590nm - SA 650 nm  $\geq$  NA590nm - NA 650nm + 0.15 where :

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16 477 SA: Sample absorbance.

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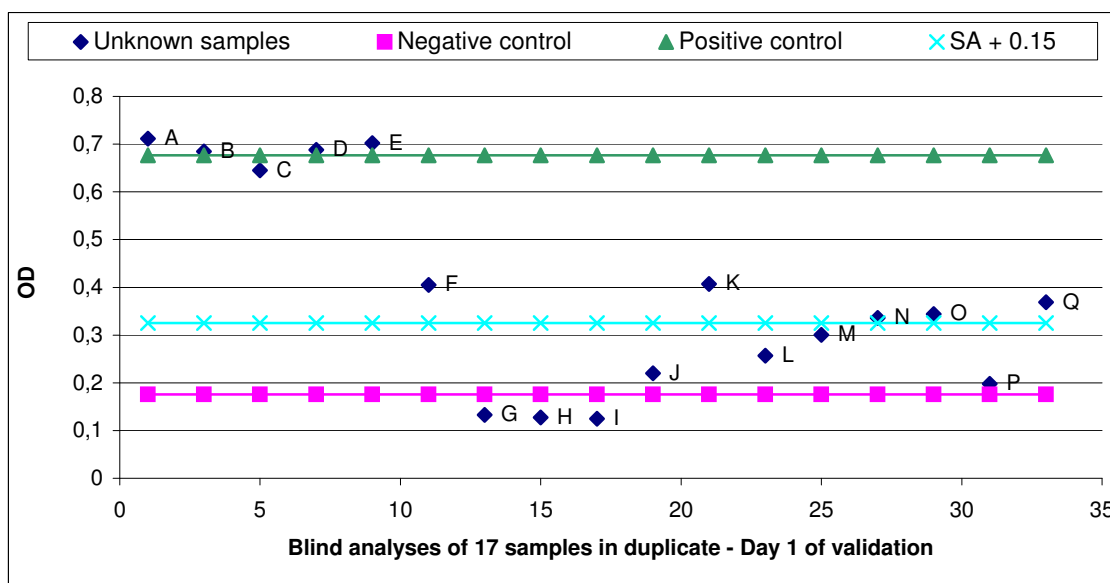
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Figure 1.



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2 1 Table I. 2-level experimental design with 4 factors: levels of each factor.  
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			Normal
4 tested factors:	Level "-"	Level "+"	conditions
A- Volume of samples	80 µl	120 µl	100 µl
B- Pre-incubation time	20 min	40 min	30 min
C- Pre-incubation temperature	62 °C	68 °C	65 °C
D- Incubation temperature	62 °C	68 °C	65 °C

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6 Table II. Robustness study: Design matrix and experimental design calculation.

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

7 <sup>a</sup> Volume of samples; <sup>b</sup> Pre-incubation time; <sup>c</sup> Pre-incubation temperature; <sup>d</sup> Incubation temperature

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9



10 Table III. Detection capabilities of the 5 tested antimicrobials (in  $\mu\text{g kg}^{-1}$ ).

Antimicrobial family	PENICILLINS		CEPHALOSPORINS		TETRACYCLINES		SULPHONAMIDES		MACROLIDES		
Antimicrobial compound	Amoxicillin		Cefalexin		Doxycycline		Sulfathiazole		Tylosin		
<b>MRL muscle (<math>\mu\text{g kg}^{-1}</math>)</b>	<b>50</b>		<b>200</b>		<b>100</b>		<b>100</b>		<b>100</b>		
Announced detection limit ( $\mu\text{g kg}^{-1}$ )*	8		400-600		150-200		100-200		80-160		
2 tested concentrations ( $\mu\text{gkg}^{-1}$ **)	10	50	200	500	100	200	100	200	100	150	
<b>Internal negative control</b>	Percentage of positive results (%)		100	100	30	85	40	100	65	100	100
	<b>CC<math>\beta</math> (<math>\mu\text{g kg}^{-1}</math>) Explorer</b>		<b>10</b>	<b>&gt;500</b>		<b>200</b>		<b>200</b>		<b>100</b>	
<b>Negative control Zeu-Inmunotec</b>	Percentage of positive results (%)		100	100	30	100	45	100	75	100	100
	<b>CC<math>\beta</math> (<math>\mu\text{g kg}^{-1}</math>) Explorer</b>		<b>10</b>	<b>500</b>		<b>200</b>		<b>200</b>		<b>100</b>	

11 \* by the manufacturer

12 \*\* for the determination of detection capabilities

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

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

15 <sup>a</sup> Volume of samples; <sup>b</sup> Pre-incubation time; <sup>c</sup> Pre-incubation temperature; <sup>d</sup> Incubation temperature

16 <sup>e</sup> Significant effect

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

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

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

FPT: Four Plate Test

D: Doubtful

NQ: Not Quantified