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Validation of a Five Plate Test, the STAR protocol, for the screening of antibiotic residues in muscle from different animal species according to the European decision 2002/657/EC

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3 1 **Validation of a Five Plate Test, the STAR protocol, for the screening**
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5 2 **of antibiotic residues in muscle from different animal species**
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7 3 **according to the European decision 2002/657/EC**
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3 **ABSTRACT**

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

INTRODUCTION

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

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

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3 89 antibiotics at or below the maximum residue limit (MRL), and a further 27 drugs could be
4 detected at levels from the MRL up to four times the MRL. The STAR protocol was at least
5 90
6 91 twice as sensitive as conventional methods for macrolides, quinolones and tetracyclines.
7
8 92 Each plate was preferentially sensitive for one or two families of antibacterials: the plate
9
10 93 *Bacillus cereus* for tetracyclines, the plate *Escherichia coli* for quinolones, the plate *Bacillus*
11 94 *subtilis* for aminoglycosides, the plate *Kocuria rhizophila* for macrolides and the plate *Bacillus*
12 95 *stearothermophilus* for sulphonamides and beta-lactams.
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14 96

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16 97 Since 2002, every analytical method used for the analysis of monitoring routine samples has
17 98 to be validated according to the European Decision 2002/657/EC (EC 2002) which concerns
18 99 the performance of analytical methods and the interpretation of results. The level of
19 100 validation of confirmatory methods is now quite satisfactory. However, very little information
20 101 is contained in that decision concerning the validation of screening methods. Two main
21 102 information are contained in the decision (EC 2002): firstly which performance characteristics
22 103 have to be determined for a screening method and secondly the following information about
23 104 the detection capability required for screening methods. Screening methods are “only those
24 105 analytical techniques, for which it can be demonstrated in a documented traceable manner
25 106 that they are validated and have a false compliant rate of < 5 % (β -error) at the level of
26 107 interest shall be used for screening purposes in conformity with Directive 96/23/EC (EC
27 108 1996). In the case of a suspected non-compliant result, this result shall be confirmed by a
28 109 confirmatory method.” Finally the difficulty is how to use this information to implement a
29 110 validation for a screening method.
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40 112 Moreover, the validation of microbiological screening methods presents specific issues,
41 113 different from physico-chemical methods which are often specific methods. Firstly, the
42 114 number of antibiotics to be validated is very high because all the antibiotics having a MRL in
43 115 the corresponding matrix should be validated. It could be also the case for newly developed
44 116 multi-residue methods by LC-MS/MS. However microbiological methods do not allow to
45 117 identify the antibiotic residue present in the sample. So each antibiotic has to be tested
46 118 independently and the number of analyses increased. Secondly, these kind of methods are
47 119 only qualitative methods, giving a response as negative, positive or doubtful. Deciding on a
48 120 sample size for qualitative inquiry can be even more difficult than quantitative because there
49 121 are no definite rules to be followed. In general, sample size depends on the nature of the
50 122 analysis to be performed, the desired precision of the estimates one wishes to achieve. The
51 123 larger your sample size, the more sure you can be that their answers truly reflect the
52 124 population. This indicates that for a given confidence level (e.g.. 95 %; β error = 5 %), the
53 125 larger your sample size (n), the smaller your confidence interval (interval estimate of a

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3 126 population parameter). For n=20 and a percentage of answers of 50% (i.e. 50 % answers
4 127 positive; 50 % answers negative), the confidence interval is equal to 22 %. For n=60 and a
5 128 percentage of answers of 50% (50 % positive; 50 % negative), the confidence interval is
6 129 equal to 13 %. A greater sample is then required to decrease the confidence interval and
7 130 therefore chance of error. Therefore from a statistical point of view, the number of samples to
8 131 be analysed should be higher than for quantitative methods. Thirdly, spiking liquid matrices
9 132 (e.g. milk, juice meat) is easy and validation could be performed on spiked samples. The
10 133 problem is specific of solid matrices like muscle. In fact, plate tests are based on the analysis
11 134 of raw muscle (slices of meat). Therefore the validation should be conducted on blank and
12 135 incurred raw muscle. However, the production of incurred materials for each antimicrobials at
13 136 one concentration would be time and money consuming. As a consequence, very few
14 137 microbiological methods have been validated in the matrix, especially in muscle and other
15 138 solid matrices according to the decision 2002/657/EC (EC 2002). A new guideline document
16 139 supplements Commission Decision 2002/657/EC regarding the validation of screening
17 140 methods. The Community Reference Laboratory (CRL) in Fougères in collaboration with the
18 141 CRL in Berlin and in agreement with the CRL in Bilthoven, and after consultation through the
19 142 NRL (National Reference Laboratories) network, has drafted this document with the purpose
20 143 to assist residue laboratories to validate screening methods. This document is now finalised
21 144 and officially published on the DGSANCO website since the 21st of January 2010
22 145 (Anonymous 2010). This guideline deals with the initial validation and also a shortened or
23 146 'abridged' validation, which under certain conditions, allows for the transfer of methods
24 147 already validated in one laboratory to a second one. The guideline proposes some
25 148 recommendations to implement a validation protocol for screening methods. It explains the
26 149 performance characteristics to be determined (specificity, detection capability, robustness,
27 150 ...) and how to determine them in practice. Moreover, the number of samples necessary to
28 151 validate a screening method is discussed. Finally, some new concepts have been introduced
29 152 in the guideline: the preparation of "simulated tissues" and a list of representative substances
30 153 to be validated. These two concepts will be detailed below in the validation protocol.

31 154
32 155 The validation conducted in this study is based on this validation guideline which is also the
33 156 internal guideline in our laboratory for the validation of screening methods. Two objectives
34 157 have been set: firstly to validate the STAR protocol for its application to the detection of
35 158 antibiotic residues in muscles from different animal species according to the decision
36 159 2002/657/EC (EC 2002); secondly to show that the European guideline for the validation
37 160 (Anonymous 2010) and based on the decision 2002/657/EC (EC 2002) was applicable to the
38 161 validation of a microbiological screening method.

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3 162 This paper will present the validation protocol and then the results of the validation of the
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5 163 STAR protocol for the screening of antibiotic residues in muscles of different animal species.
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8 165 **MATERIAL AND METHODS**

9 166 ***Chemicals and standard solutions***

10 167 Antibiotic and sulphonamide standards were provided by Sigma, except cefquinome
11
12 168 (Intervet), ceftiofur (Upjohn), enrofloxacin (Bayer), doxycycline (Virbac).
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14 169

15 170 ***STAR protocol***

16 171 The STAR protocol is a Five Plate Test which was already published for the analysis of milk
17
18 172 (Gaudin et al. 2004). Five test organisms (*Bacillus subtilis* B.G.A spores (MERCK), *Kocuria*
19
20 173 *rhizophila* ATCC 9341 (Pasteur Institute, France), *Bacillus cereus* ATCC 11778 (Pasteur
21
22 174 Institute, France), *Escherichia coli* ATCC 11303 (Pasteur Institute, France), *Bacillus*
23
24 175 *stearothermophilus* ATCC 10149 (MERCK)) were inoculated in 5 different media. The 5
25
26 176 following culture media were used respectively: Antibiotic medium II at pH 8.0 (plate Bs8),
27
28 177 Test agar at pH 8 (MERCK) (plate Kv8), Test agar at pH 6 (MERCK) (plate Bc6), Test agar
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30 178 at pH 8 (MERCK) (plate Ec8) and Diagnostic Sensitive Test (DST) (OXOID commercialised
31
32 179 by UNIPATH LTD, Basingstoke, Hampshire, UK) (plate Bst). Culture media were prepared
33
34 180 as recommended by the supplier and sterilised. Then 5 ml of inoculated medium were added
35
36 181 on a Petri dish placed on a cold horizontal surface. In routine use, a cylindrical plug of 8 mm
37
38 182 in diameter and 2 cm long is cut in frozen muscle using a cork borer. Then, slices of muscle
39
40 183 samples of 2 mm in thickness are cut and put on the plates. The same protocol was applied
41
42 184 to “simulated tissues”. Finally the plates are incubated: at 30°C for at least 18 hours for Bs8
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44 185 and Bc6, at 37°C for at least 24 hours for Kv8, at 37°C for at least 18 hours for Bc6 and at
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46 186 55°C for 15 to 16 hours for Bst.
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49 188 A muscle sample was considered positive when the inhibition zone around meat sample was
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51 189 equal or superior to 2 mm in width on plates Bs 8, Kv 8, Bc 6 and Ec 8 and / or the inhibition
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53 190 zone equal or superior to 4 mm in width on plate Bst.
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56 192 Since 2004, medium test agar pH 7.2 has been replaced by antibiotic medium II at pH 8.0
57
58 193 (Difco, reference 259310) because it was not commercialised anymore.. Moreover, the
59
60 194 positive control of plate Bst was modified for practical reasons: sulfamethazine at 1000 µg l⁻¹
195 has been replaced by amoxicillin at 40 µg l⁻¹. Positive controls consist of 30 µl of antibiotic
196 solutions which are put on paper discs of 9 mm diameter (Durieux, France). There is a
197 specific positive control for each plate: streptomycin at 2000 µg l⁻¹ on plate Bs8, tylosin at
198 1000 µg l⁻¹ on plate Kv8, oxytetracycline at 800 µg l⁻¹ on plate Bc6, enrofloxacin 800 µg l⁻¹ on

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3 199 plate Ec8 and amoxicillin at 40 $\mu\text{g l}^{-1}$ on plate Bst. The validity of each day of analysis
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5 200 depends on the results of the positive controls which have to be included in the following
6
7 201 intervals: 5.5 \pm 1.5 mm, 6.5 \pm 1.5 mm, 6.0 \pm 1.5 mm, 7.0 \pm 1.5 mm and 6.0 \pm 1.5 mm for Bs8,
8
9 202 Kv8, Bc6, Ec8 and Bst respectively.
10

11 204 **Validation protocol**

13 205 *Simulated tissues*

14 206 In 2002, we have studied the sensitivity of 35 antibiotics by the way of antibiotic spiked
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16 207 discs. However, this way of working was not completely satisfying because the interference
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18 208 of the muscle matrix was absent. In fact, Okerman et al (1998a) showed that the tissue
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20 209 matrix has an effect on the sensitivity of the test plates. During that study, pieces of frozen
21
22 210 meat laid on paper discs impregnated with antibiotic standard solutions have been used for
23
24 211 the validation. Usually inhibition zones decreased when spiked meat samples have been
25
26 212 analysed, compared to antibiotic spiked discs without meat. The same conclusions were
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28 213 reported by Pikkemaat et al. (2007). Because of the difficulty and the high cost of
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30 214 production of incurred materials for validation of a microbiological plate test, it was decided
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32 215 to work on what we called "simulated tissue. Furthermore, it was impossible to mix several
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34 216 antibiotics in the same sample, since the method was not specific and did not identify the
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36 217 molecule that produced an inhibitory effect. Moreover, one could observe a cumulative
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38 218 effect of antibiotics in their inhibitory activity when several antibiotics are contained in one
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40 219 sample.
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43 221 Therefore, a preliminary study was conducted to determine what was the best way to
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45 222 prepare simulated tissues and what kind of preparation would give the closest result to the
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47 223 actual samples. This study was based on the experience of 2 National Reference
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49 224 Laboratories (Vicente Calderon, AESAN, Spain; Anna Liisa Myllyniemi, EVIRA, Finland)
50
51 225 that had already worked on such samples. During this preliminary study, the way of
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53 226 preparation of simulated tissue was also tested for homogeneity of the material. Regarding
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55 227 the results, the homogeneity was satisfactory.
56

57 228 58 229 *Production of spiked materials ("Simulated tissue")*

59 230 A preliminary study has been conducted to determine the concentrations of antibiotics to be
60
231 spiked in muscle samples for later validation. Sixteen different antibiotics have been tested at
232 various concentrations (the MRL and according to the assumed detection limit at half MRL, 2
233 times the MRL or more) in simulated tissues. The tested concentrations were based on the
234 results of the validation of the STAR protocol in milk (Gaudin et al. 2004). These tests were
235 repeated several times. The concentration which always gave positive results (inhibition

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3 236 zones higher than 2 mm, generally between 3 and 4 mm) and if possible concentrations
4 237 lower than or equal to the respective MRLs have been chosen.
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6 238 The antibiotics and the corresponding concentrations chosen for the validation are presented
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8 239 in Table 1.
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10 240

11 241 **Insert table 1 about here**

12 242

13
14 243 Sixteen different antibiotics were used in this study. Nine different groups of antibiotics were
15 244 studied : macrolides, aminoglycosides, cephalosporins, penicillins, quinolones, tetracyclines,
16 245 sulphonamides, lincosamides and miscellaneous drugs. Stock solutions of the 16 different
17 246 antimicrobials were prepared at a concentration of 1 mg ml⁻¹, after correction of potency.
18 247 Then working solutions were prepared by dilutions in distilled water. Different batches of
19 248 muscle were purchased in supermarkets. Muscle was firstly coarsely minced. To prevent
20 249 antibiotic contamination, finely minced blank muscle samples were prepared first, the same
21 250 day, with the 4 different batches of muscle, to be tested in parallel with the spiked samples.
22 251 Then 1 ml of working solution was added to 100 g of blank minced muscle. Spiked muscle
23 252 was homogenised during 15 minutes in the same rotary hatcher. At the end, the meat is
24 253 finely minced. Each muscle material was finally put in plastic bottles or plastic bags. Each
25 254 material was codified by the director of the study and then was frozen at -20°C. The
26 255 "simulated tissues" were always analysed in a maximum period of 1 month.
27

28 256

29 257 **Specificity**

30 258 49 batches of pork muscle of different origins were analyzed in the end. Most of them (40
31 259 batches) have been tested in blind duplicate on 2 different days and by 2 different
32 260 technicians.
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34 261

35 262 **Detection capabilities CC β**

36 263 According to EU Commission Decision 2002/657/EC (EC 2002), the detection capability
37 264 (CC β) of a method is defined as "the smallest content of the substance that may be
38 265 detected, identified and/or quantified in a sample with an error probability of β . In the case of
39 266 substances with an established permitted limit, this means that the detection capability is the
40 267 concentration at which the method is able to detect permitted limit concentrations with a
41 268 statistical certainty of $1 - \beta$ ".
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43 269

44 270 Given the expected detection capabilities for many antibiotics, which are often close to the
45 271 MRLs, it was chosen to determine the detection capabilities by analyzing 60 minced muscle

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3 272 samples spiked at one antibiotic concentration, instead of 20 samples to reach the most
4 273 statically significant determination of the $CC\beta$ (EC 2002).

5 274

6 275 **Insert Figure 1 about here**

7 276

8 277 The preparation of the samples is detailed in figure 1. To introduce a maximum of variability
9 278 in the determination of the detection capabilities, 30 samples have been prepared and
10 279 analyzed by one technician and another 30 samples by other technician. In addition, the 30
11 280 samples have been divided into 2 sets of preparation. In each set, 15 samples for the same
12 281 antibiotic concentration have been prepared and analyzed by each technician. For each
13 282 antibiotic and each technician, the 2 sets have been spaced out, to introduce variability in the
14 283 period of analysis (ambient conditions) and therefore variability of batches (media, bacteria,
15 284 antibiotic standard...). Moreover, in each set of preparation of 4 antibiotics, 4 different
16 285 batches of porcine muscle have been used. Finally, on each day of preparation, 2 different
17 286 working solutions have been prepared from the stock solution of antibiotic and used to spike
18 287 the different batches of muscle. The coding of the samples has been performed by the head
19 288 of the study, to ensure that the analyses would be performed blindly, and then frozen at -20
20 289 °C.

21 290

22 291 In the determination of specificity and detection capabilities, all samples have been analyzed
23 292 on the 5 plates of the STAR protocol to check the specificity of the plates for the different
24 293 families of antibiotics. Five days of analyses have been performed with the STAR protocol for
25 294 each set of preparation and each technician. Each day, 14 samples have been analysed
26 295 blindly (12 spiked samples and 2 blank samples). Therefore, all blank and spiked samples
27 296 have been analyzed twice or 3 times, on 2 or 3 different days.

28 297

29 298 One wants to underline that the analysis of the 60 samples for each antimicrobial were
30 299 performed step by step. When one false negative result or more was obtained after the
31 300 analysis of 10 samples, either the validation was stopped at this concentration and started
32 301 again with an increased concentration if it was of interest (e.g. near the MRL) or the
33 302 validation went on with the same antibiotic and the same concentration because the
34 303 concentration was already much higher than the concerned MRL.

35 304

36 305 Determination of $CC\beta$: After the analysis of the 60 spiked samples, the concentration level,
37 306 where only less than 5 % of false compliant results remains was the detection capability $CC\beta$
38 307 of the method (3 false compliant results maximum out of 60 spiked samples).

39 308

309 **Applicability study**

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

316
317 However, to complete this study, we decided to perform a study to determine the CC β of
318 some antibiotics in cattle, sheep and poultry muscles, antibiotics for which the CC β were
319 determined previously in porcine muscle. Therefore, the CC β of 5 antimicrobials (penicillin G
320 at 25 $\mu\text{g kg}^{-1}$, doxycycline at 100 $\mu\text{g kg}^{-1}$, erythromycin at 400 $\mu\text{g kg}^{-1}$, gentamicin at 6000 μg
321 kg^{-1} , enrofloxacin at 200 $\mu\text{g kg}^{-1}$) have been determined with 20 samples from each of the 3
322 species over 5 days and have been compared with the CC β calculated for porcine muscle. In
323 the applicability study, only the specific plate (which has presented inhibition zones for pig
324 muscle) was tested with the corresponding antibiotic.

325
326 The applicability would be proved if the CC β determined for these species are similar to the
327 CC β determined for porcine muscle (the average inhibition zones obtained for each species
328 on the specific plate should be similar: accepted deviation $\pm 25\%$). A variability of 25 % is
329 usually accepted with microbiological methods (for positive controls or spiked muscles).
330 Moreover, this range of variability was observed during the validation in porcine muscle. The
331 specificity of the STAR protocol with other species has also been studied by the analysis of
332 different blank muscle samples.

333

334 **Ruggedness study**

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

339

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

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3 345 chosen for the analysis of the effects and interactions of 4 independent factors (Renard et al.
4 346 1992). The design matrix is presented in Table 2.

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8 348 **Insert Table 2 about here**

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11 350 Factors which may influence the measurement results have been selected: bacteria
12 351 concentration (A), medium quantity (B) in the plate, incubation time (C) and pre-incubation
13 352 time at room temperature (D). These factors have been modified in an order of magnitude
14 353 corresponding to the usual differences: factor A concentration in bacteria: $\pm 30\%$; factor B
15 354 quantity of medium 5 ± 0.5 ml; factor C incubation time $\pm 10\%$; factor D pre-incubation time
16 355 (period at room temperature before incubation in the incubator: 1 hour of pre-incubation or no
17 356 pre-incubation).

18
19 357

20 358 The ruggedness study has been focused on 6 different representative antimicrobials which
21 359 were specifically detected on each of the 5 plates on muscle: penicillin G at $25 \mu\text{g kg}^{-1}$ and
22 360 sulfadimethoxine at $300 \mu\text{g kg}^{-1}$ on plate Bst, doxycycline at $100 \mu\text{g kg}^{-1}$ on plate Bc6,
23 361 erythromycin at $400 \mu\text{g kg}^{-1}$ on plate Kv8, gentamicin at $6000 \mu\text{g kg}^{-1}$ on plate Bs8,
24 362 enrofloxacin at $200 \mu\text{g kg}^{-1}$ on plate Ec8. The chosen concentrations were equal to the
25 363 detection capabilities determined in the first part of the validation study for each of these
26 364 antibiotics, except for gentamicin. The samples were prepared from pig muscle only. All
27 365 blank and spiked minced muscle samples were prepared the day before starting the
28 366 ruggedness study and were frozen at -20°C . The study was performed blindly (codified
29 367 samples). Then each day of analysis (each run), 4 different blank materials and 4 different
30 368 spiked materials per antibiotic were analysed.

31
32 369

33 370 **RESULTS AND DISCUSSION**

34 371 ***Specificity***

35 372 Specificity and detection capabilities were determined for pig matrix because it is the most
36 373 common species analyzed in the laboratory. 49 different batches of porcine muscle have
37 374 been tested. Few variations have been observed. Of 176 analyses in total, only 7 gave false
38 375 positive results (4%). The 7 false positive results were obtained with 5 different muscle
39 376 batches (2 false positive results for 2 batches and 1 false positive result for each of 3
40 377 batches). All false positive results have appeared on the plate Bst, none on the other plates.
41 378 The specificity of the STAR protocol for the detection of antimicrobial residues in porcine
42 379 muscle was very satisfactory.

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3 381 **Detection capabilities**

4 382 The results presented in table 3 are those obtained from 60 samples for each antibiotic at
5 383 least one tested concentration.

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10 385 **Insert Table 3 about here**

11 386

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13 387 For some antibiotics, after 15 or 30 analyzes by one or two technicians, either it turned out
14 388 that too many false negative results were obtained at the selected concentration or on the
15 389 opposite the first tested concentration gave higher inhibition zones than anticipated,
16 390 suggesting that the validation could be performed at a lower concentration. At these
17 391 concentrations, less than 60 samples have been analyzed, however it was helpful for the
18 392 determination of the detection capability.

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22 394 Figure 2 is a graphical representation of the relationship between detection capabilities
23 395 calculated for the 16 representative antibiotics and their respective MRLs.

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26 397 **Insert Figure 2 about here**

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29 399 This figure allows one to quickly visualize the difference between $CC\beta$ and MRLs. The
30 400 respective MRLs ($\mu\text{g kg}^{-1}$) are represented in plain bars. The hatched bars represent the
31 401 respective $CC\beta$ ($\mu\text{g kg}^{-1}$). Therefore, the highest is the hatched area, the least sensitive is the
32 402 STAR protocol for the corresponding antibiotic. On the opposite, when plain zone and
33 403 hatched zone are very near, the sensitivity of the method for the corresponding antibiotic is
34 404 satisfactory. When the $CC\beta$ is higher than $1000 \mu\text{g kg}^{-1}$, the value of the $CC\beta$ is written on
35 405 the top of the corresponding bar.

36 406

37 407 As a conclusion, the detection capabilities of penicillin G, cloxacillin and doxycycline were
38 408 equal to or lower than their respective MRLs. The detection capabilities of tylosin,
39 409 sulfadimethoxine, oxytetracycline, trimethoprim, erythromycin, ceftiofur, enrofloxacin and
40 410 cefquinome were between 1.5 and 3 times their respective MRLs. The detection capability of
41 411 lincomycin was between 4 and 5 times its MRL. Finally, the detection capabilities of
42 412 gentamicin (GTM) ($> 40 \times \text{MRL}$), dihydrostreptomycin (DHS) ($> 8 \times \text{MRL}$) and florfenicol ($>$
43 413 $10 \times \text{MRL}$), were much higher than their respective MRLs. The STAR protocol is not suitable
44 414 for the detection of the 2 aminoglycosides and probably not for the detection of the entire
45 415 family, given the activity profiles obtained during the validation in milk (Gaudin et al. 2004). In
46 416 fact, these activity profiles showed that GTM and DHS were the 2 aminoglycosides which
47 417 were detected at the lowest concentrations, especially GTM. The sensitivities for GTM in milk

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3 418 (MRL = 100 $\mu\text{g l}^{-1}$) and to DHS (MRL = 200 $\mu\text{g l}^{-1}$) were equal to 3 and 5 times the respective
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5 419 MRLs. The sensitivities determined in milk for the other aminoglycosides were as follows:
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7 420 neomycin 1500 $\mu\text{g l}^{-1}$ (MRL = 1500 $\mu\text{g l}^{-1}$), streptomycin 1000 $\mu\text{g l}^{-1}$ (MRL = 200 $\mu\text{g l}^{-1}$),
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9 421 kanamycin 1000 $\mu\text{g l}^{-1}$ (MRL = 150 $\mu\text{g l}^{-1}$), spectinomycin 40000 $\mu\text{g l}^{-1}$ (MRL = 300 $\mu\text{g l}^{-1}$),
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11 422 paromomycin 2000 $\mu\text{g l}^{-1}$ (not authorised in milk), apramycin 4000 $\mu\text{g l}^{-1}$ (not authorised in
12
13 424 milk). Therefore, the sensitivities for GTM and DHS were much better in milk than in muscle.

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15 425 During the determination of detection capabilities in porcine muscle, it was demonstrated that
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17 426 the STAR protocol could provide an help to confirm the identity of a family of molecules
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19 427 present in the sample. Table 4 shows the plates on which each antibiotic reacted
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21 428 preferentially. Some antibiotics (e.g. cefquinome, ceftiofur, doxycycline, enrofloxacin and
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23 429 florfenicol) caused inhibitions onto several plates at the tested concentrations (including
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25 430 $\text{CC}\beta$).

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27 432 **Insert Table 4 about here**

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29 434 The results were obviously similar to the global orientation given in the STAR protocol: beta-
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31 435 lactams and sulphonamides were detected preferentially on plate Bst, tetracyclines on plate
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33 436 Bc6, quinolones on plate Ec8 and macrolides on plate Kv8. However, there were 2
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35 437 exceptions. The aminoglycosides have been detected on plate Bst with the concentrations
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37 438 tested for the determination of $\text{CC}\beta$, whereas for higher concentrations than $\text{CC}\beta$, they were
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39 439 detected on plate Bs8 = Bs7.2). However, when the validation of the STAR protocol has
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41 440 been performed in milk, Bs8 was the specific plate for the detection of aminoglycosides.
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43 441 Secondly, tylosin was detected preferentially on Bst instead of Kv8 at this concentration,
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45 442 which had already been observed during the validation of the STAR protocol in milk. So if a
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47 443 positive result is reported on plate Bst, the confirmation should be directed onto beta-
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49 444 lactams, sulphonamides, but also tylosin and aminoglycosides.

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51 446 **Applicability study**

52 447 Given the unsatisfactory results for aminoglycosides (\gg MRL), an additional study was
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54 448 conducted on GTM and DHS to choose one of these antibiotics and the good concentration
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56 449 for applicability and ruggedness studies. This study determined that the detection capability
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58 450 of GTM was around 4000 $\mu\text{g kg}^{-1}$ and DHS around 6000 $\mu\text{g kg}^{-1}$. Therefore, GTM at 6000 μg
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60 451 kg^{-1} has been chosen, a concentration higher than the estimated detection capability (4000
452 $\mu\text{g kg}^{-1}$) due to reading difficulties (Partial Inhibition Zone (PIZ) and regrowth in the inhibition
453 zone).

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3 454 Figure 3 represents the average inhibition zones on the 5 different plates (Bst, Bc6, Kv8, Ec8
4 and Bs8) for blank muscles of different animal species and muscles spiked with 5 different
5 455 antibiotics (each one reacted specifically on one of the plates) : penicillin G at $25 \mu\text{g kg}^{-1}$ on
6 456 Bst (figure 3a), doxycycline at $100 \mu\text{g kg}^{-1}$ on Bc6 (figure 3b), erythromycin at $400 \mu\text{g kg}^{-1}$ on
7 457 Bst (figure 3a), doxycycline at $100 \mu\text{g kg}^{-1}$ on Bc6 (figure 3b), erythromycin at $400 \mu\text{g kg}^{-1}$ on
8 458 Kv8 (figure 3c), enrofloxacin of $200 \mu\text{g kg}^{-1}$ on Ec8 (figure 3d) and gentamicin at $6000 \mu\text{g kg}^{-1}$
9 459 on Bs8 (figure 3e). The results for the porcine muscle were those obtained during the
10 460 determination of CC β for 4 plates (Bst, Bc6, Kv8, Ec8) and during the additional study on
11 461 aminoglycosides for plate Bs8.
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18 463 **Insert Figure 3 about here**
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21 465 The overall average inter-species was equal to 9.0 ± 1.3 mm, 5.2 ± 1.0 mm, 5.2 ± 1.4 mm,
22 466 6.1 ± 1.1 mm and 5.4 ± 0.9 mm for Bst, Bc6, Kv8, Ec8 and Bs8 respectively. Whichever was
23 467 the tested antibiotic and the plate, the discrimination between blank and spiked samples was
24 468 very clear and easy, for each species. Moreover, the average inhibition zone (IZ) for cattle,
25 469 sheep and poultry muscle was in the fixed interval of plus or minus 25% compared to the
26 470 average IZ for porcine muscle for plates Bst, Bc6, Ec8 and Bs8. Concerning plate Kv8, the
27 471 average inhibition zone (IZ) for cattle and sheep muscle was in the fixed interval of plus or
28 472 minus 25% compared to the average IZ for porcine muscle. The poultry muscle was out of
29 473 the interval for plate Kv8, but with an average inhibition zone of 33% higher than the porcine
30 474 muscle, so the sensitivity was better in the poultry muscle.
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39 476 Two blank samples of cattle, 2 sheep and 2 poultry were analyzed for each plate and the
40 477 analyses were repeated for 5 days. Over 10 cattle tested, we got 2 false-positive results, 1
41 478 for sheep and 1 for poultry on Bst plate. The blank bovine and ovine samples gave IZ of 3.7
42 479 ± 0.7 and 3.3 ± 0.4 mm, higher than porcine (1.8 ± 1.4 mm) and poultry (2.0 ± 1.4 mm) blank
43 480 samples. All blank samples gave non specific inhibition on the plate Bst, higher than for the
44 481 other plates. That is why the positivity threshold of the plate Bst was set at 4 mm, while the
45 482 positivity threshold for the other plates was set at 2 mm (Fuselier at al. 2000). No false-
46 483 positive results were obtained on the plate, whatever the species, on the 4 other plates.
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52 484 In conclusion, the STAR protocol is applicable for the screening of antibiotic residues in the
53 485 muscle of the 4 major species: pig, cattle, sheep, poultry and by extension the muscle of
54 486 minor species, whatever is the plate.
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3 489 ***Ruggedness study***

4 490 During the determination of specificity and detection capabilities of the 16 antimicrobials, lots
5 491 of different media (different preparation and validity date), different preparations of bacteria,
6 492 different batches of antibiotic standards have been used over a period of 9 months of testing.
7 493 Looking at the quantitative results (inhibition zones) obtained by the 2 technicians on muscle
8 494 samples, , the variability (standard deviations) was rather low, similar to or lower than the
9 495 variability that is fixed for the positive controls of the method. During this period, the positive
10 496 controls were included in the intervals set in the STAR protocol. This is the first track to
11 497 conclude that the STAR method is robust.
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19 499 The average inhibition zones and the standard deviations obtained during the ruggedness
20 500 study and during the first part of the validation (determination of $CC\beta$) have been compared
21 501 and are presented in Table 5.
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26 503 **Insert Table 5 about here**

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29 505 When the standard deviation in ruggedness conditions (SDi) is significantly larger than the
30 506 standard deviation of the method in terms of reproducibility, the conclusion is predictable that
31 507 all factors taken together have an impact on the results, even if no single factor has
32 508 significant influence. In this case, the method is not sufficiently robust regarding the range of
33 509 tested variations. From a quantitative point of view, we did not observe significant differences
34 510 between the SD in ruggedness conditions and the SD during the first part of the validation for
35 511 the 6 tested antibiotics. Therefore, the STAR protocol seemed to be robust for the 5 plates
36 512 regarding the range of tested variations.
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44 514 If we look at the mean inhibition zones obtained at each day (run) during the ruggedness
45 515 study to the mean inhibition zones obtained during the determination of detection
46 516 capabilities, it seemed that the sensitivity is very near, even if the average inhibition zones
47 517 were often lower during the ruggedness study. In fact, the average inhibition zone of SDMX
48 518 on plate Bst was significantly lower during the ruggedness study (Table 5). Therefore some
49 519 of the tested factors had an effect on the sensitivity. Sulphonamides tested concentrations
50 520 often gave Partial Inhibition Zones (PIZ), which are sometimes quite tricky to read for
51 521 someone not trained. On the opposite, beta-lactams usually gave clear inhibition zones. So,
52 522 slight variations like in the ruggedness study, even for a trained people, led to more effect on
53 523 these PIZ than on clear inhibition zones. Therefore, it is logical that the Bst plate is less
54 524 robust for the detection of SDMX than for penicillin G.
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3 525 The ruggedness of the STAR method has been evaluated using an experimental design. The
4 526 influence of the 4 factors (concentration of bacteria, quantity of medium, incubation time and
5 527 pre-incubation time) on the mean inhibition zone (quantitative result), repeatability (coefficient
6 528 of variation CV%) and false positive and false negative rate (qualitative result), as well as the
7 529 interactions between factors, have been evaluated (Table 2). The responses for each plate
8 530 for each run (day) were indicated (mean inhibition zone (IZ), the coefficient of variation (CV
9 531 %) on the inhibition zone and false positive and false negative rates when influence was
10 532 observed). From these results, the exploitation of the experimental design has been
11 533 performed. The results are presented in Table 6 for the 6 antibiotics and the 5 plates.
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19 535 **Insert Table 6 about here**
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23 537 In conclusion, whatever the plates, there was no significant effect of analytical factors tested
24 538 on the qualitative results of the STAR method (no influence on the false-positive rate and
25 539 false-negative rate), except for the plate Bst with SDMX (**Table 6**). When testing SDMX, on
26 540 day 4, the 4 tested samples gave false-negative results (**Table 7**). Therefore it has a strong
27 541 impact of the combination of factors tested that day: A +, B +, C-, D-, i.e. increasing the
28 542 concentration of bacteria, increasing the amount of medium, reducing the incubation period
29 543 and without pre-incubation. In the optimization of microbiological methods, it is well known
30 544 that increasing the concentration of bacteria or increasing the amount of medium may
31 545 decrease the sensitivity of the plate. Moreover, as seen with other plates, a decrease of the
32 546 incubation period can indeed reduce the sensitivity. It is also logical that a lack of pre-
33 547 incubation may decrease the sensitivity of the plate. It was observed in fact that an increase
34 548 of factors A and B may increase the false negative rate, while an increase of the incubation
35 549 time and pre-incubation of one hour may increase the sensitivity. It is noticeable that 1 hour
36 550 of pre-incubation at room temperature increased the mean inhibition zone of SDMX of 17%.
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47 552 **Insert Table 7 about here**
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51 554 Regarding the classical variability of results of microbiological plate tests, none of the factors
52 555 or combination of factors had significant effect on the mean inhibition zone (quantitative
53 556 result) for any of the tested antibiotics on the 5 plates. Finally, no significant effect has been
54 557 observed on the repeatability of the method. Moreover, slight changes in the coefficient of
55 558 variation never had effect on the qualitative results of the test for the tested antibiotics.
56 559 Therefore, the STAR protocol is a robust method for the detection of antibiotic residues in
57 560 muscle. Some recommendations are given in the STAR protocol, concerning the different
58 561 incubation periods for the 5 plates, because incubation time was already known as a critical
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3 562 parameter. In the range recommended in the protocol, the method is robust. It should be
4 563 noticed that increasing the incubation time for plate Kv8 increased the sensitivity of the plate
5 564 of 13 % (**Table 6**). This is the reason why a longer incubation time (at least 24 hours)
6 565 compared to the other plates is recommended in the STAR protocol. However, even at the
7 566 lowest incubation time (21 hours), the results were satisfactory.
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11 568 Finally, it has been demonstrated that a pre-incubation of 1 hour at room temperature could
12 569 have a positive effect on the sensitivity of the test (plates Ec8, Bst (SDMX), Kv8) or no effect
13 570 (plates Bc6, Bs8, Bst (penicillin G)), compared to no pre-incubation. However, pre-incubation
14 571 is not a critical parameter because if people does not apply a pre-incubation, the results
15 572 would be satisfactory and if pre-incubation is performed, the results would be equal or better.
16 573 A recommendation of pre-incubation 1 hour at room temperature, should be added in the
17 574 next version of the STAR protocol. Moreover, every day of analysis, specific positive control
18 575 antibiotic paper discs are put on each plate. The results of these positive controls should be
19 576 included in the range given in the STAR protocol. If it is the case, the results are valid.
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29 577 30 578 **Discussion**

31 579 At this time, very few laboratories tried to validate their screening methods (microbiological or
32 580 immunological methods) according to the decision 2002/657/EC (EC 2002) for 2 main
33 581 reasons. Firstly, the decision 2002/657/EC (EC 2002) gave very little information and no
34 582 technical recommendations for the implementation of the validation of a screening method.
35 583 Secondly, especially for the validation of microbiological plate tests, the validation requires a
36 584 long work, is time-consuming and quite expensive. In our laboratory, which is the Community
37 585 Reference Laboratory for antibiotic residues, the validation of screening methods has been a
38 586 subject of interest for many years. At this time, the validation of immunological tests and
39 587 microbiological tests (tube tests and plate test) have been performed in our laboratory
40 588 according to the European decision 2002/657/EC (Gaudin et al. 2004; Gaudin et al. 2007;
41 589 Gaudin et al. 2009a; Gaudin et al. 2009b). The former "detection limit" has been replaced by
42 590 a new performance characteristic called "detection capability CC β ". To our knowledge, the
43 591 other validation studies of microbiological plate tests according to the European decision (EC
44 592 2002) were from a National Reference Laboratory in the Netherlands (Pikkemaat et al. 2007;
45 593 Pikkemaat et al. 2008; Pikkemaat et al. 2009b).
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58 595 Regarding the time needed for this validation study, it would have been impossible to
59 596 validate the STAR protocol for all the antibiotics having a MRL in muscle matrix (more than
60 597 50 antibiotics). The work of validation would be too long, expensive and laborious. Therefore

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3 598 the recommendation to use a list of representative antibiotics was a very good compromise
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5 599 and allowed to reduce drastically the scope of the validation. It has been proposed since the
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7 600 first version of the European guideline for the validation of screening method in 2005 to
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9 601 validate a wide range test only for a list of representative antibiotics. It was adopted in the
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11 602 final version of the guideline (Anonymous 2010). The choice of the representative antibiotics
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13 603 is not fixed and is dependant of different factors: the activity patterns of different antibiotics in
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15 604 one family, the matrix, the use of antibiotics in one specific country, the assumed sensitivity
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17 605 of the method towards some antibiotics. The first step is to conduct a preliminary study that
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19 606 should allow to determine a common pattern of activity for one family or at least several
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21 607 substances of the family on a specific class of bacteria. Therefore one compound could be
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23 608 chosen to be representative of the other substances of the family in term of activity profile on
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25 609 bacteria. Then different antibiotics should be chosen for example for milk or muscle matrices.
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27 610 In fact the antibiotics used for intra-mammary treatment or for oral use could be different.
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29 611 Therefore, the interest of one antibiotic only used as intra-mammary treatment is very limited
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31 612 for a validation in muscle. Furthermore, if some antimicrobials are not used or not registered
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33 613 in some countries, there is no interest to validate for this compound if the method is intended
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35 614 to a national control. Finally, the selected analytes are dependent of the analytical method.
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37 615 So if the method to validate clearly badly detect one of antimicrobial, it is needless to
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39 616 determine its detection capability because this antibiotic would not be included in the scope
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41 617 of the method.

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45 619 A similar proposition of validating for a list of representative antibiotics was made by
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47 620 Pikkemaat et al. (2009b). After the determination of the activity profiles of 36 antibiotics for
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49 621 the NAT-post-screening test for the detection of antibiotic residues in kidney, the authors
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51 622 suggested that the validation could be performed on a list of representative antibiotics to
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53 623 reduce the scope of validation for routine field laboratories for example.

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57 625 One list of representative antibiotic was initially included as an example in the guideline
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59 626 which was extracted from the validation of the STAR protocol in milk (Gaudin et al. 2004). In
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61 627 fact, activity patterns have been determined for 66 antimicrobials having MRL in milk. The
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63 628 conclusion was that several antibiotics in one family could be gathered into one group
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65 629 because they showed similar activity profiles (same specific plate, similar sensitivity). Each
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67 630 family could be divided into 2 or more groups. Finally, 1 or 2 antibiotics per family have been
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69 631 chosen because its (or their) activity pattern (s) was (were) representative of one (or 2)
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71 632 group(s) of antimicrobials in the same family. Compared to this list of representative
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73 633 antibiotics, some molecules were replaced by others during the validation study of the STAR
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75 634 protocol, for the following reasons:

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3 635 - Some antibiotics are only used as intra-mammary treatment (e.g. cefalonium has
4 636 been replaced by ceftiofur because ceftiofur is widely used in cattle and swine to treat
5 637 respiratory diseases while cefalonium is only administered intra-mammary),
6 638 Moreover, it was determined during the validation of the STAR protocol in milk
7 639 (Gaudin et al. 2004) that the least detected cephalosporins were cefquinome (MRL =
8 640 50 $\mu\text{g l}^{-1}$) and cefalexin (MRL = 200 $\mu\text{g l}^{-1}$). On the opposite, the best detected
9 641 cephalosporins were cefazolin (Annex II for all tissues except milk) and cephapirin
10 642 (MRL = 50 $\mu\text{g l}^{-1}$). Finally, ceftiofur (MRL = 1000 $\mu\text{g l}^{-1}$) was better detected than
11 643 cefquinome but less detected than cefazolin. Therefore, we have chosen to validate
12 644 for ceftiofur and cefquinome because one was the least detected antibiotic of the
13 645 family, with a low MRL (cefquinome) and the other (ceftiofur) has an intermediary
14 646 detection with a high MRL. Regarding the activity profiles on the STAR protocol, it
15 647 was assumed that if cefquinome is detected at 2000 $\mu\text{g kg}^{-1}$ (CCbeta), cefazolin and
16 648 cephapirin would be easily detected.
17 649
18 650 - The antibiotic should be largely used in the country of implementation of the method
19 651 (e.g. in France, sulfamethazine replaced sulfathiazole). Moreover, colistin which
20 652 belongs to the polymyxin family has been replaced by a second macrolide: tylosin
21 653 because macrolides are often used for animal treatment (cattle, swine, poultry).
22 654
23 655 - During the development of the STAR protocol, it was already shown that the method
24 656 had a very poor sensitivity for some antibiotics (e.g. flumequine, sulfaguanidine,
25 657 colistin and spectinomycin), largely above the respective MRLs. So
26 658 dihydrostreptomycin (DHS) replaced spectinomycin (level of detection in milk: 20000
27 659 to 40000 $\mu\text{g l}^{-1}$). During the validation of the STAR protocol in milk (Gaudin et al.
28 660 2004), the level of detection of colistin was included between 200 and 2000 times its
29 661 MRL (MRL 50 $\mu\text{g l}^{-1}$ in milk and 150 $\mu\text{g l}^{-1}$ in muscle). Therefore the STAR protocol is
30 662 not fitted to the detection of colistin or of spectinomycin.
31 663
32 664 - Some antibiotics are frequently detected in routine monitoring samples: the
33 665 tetracyclines, particularly oxytetracycline (OTC). Thus, tetracycline has been replaced
34 666 by oxytetracycline and doxycycline was added instead of flumequine. During the
35 667 validation of the STAR protocol in milk, the limits of detection for OTC and TTC were
36 668 both equal to 250 $\mu\text{g l}^{-1}$ (Gaudin et al. 2004). The detection limits of doxycycline (50 μg
37 669 l^{-1}) and CTC (75 $\mu\text{g l}^{-1}$) were better. Therefore tetracycline family has been divided in
38 670 2 groups from which OTC (the least detected) and doxycycline (the best detected)
39 671 were chosen as representative compounds.

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3 672 In a similar way, penicillin and cloxacillin have been chosen as the representative compound
4 for the penicillin family because penicillin G (LOD 5 $\mu\text{g l}^{-1}$) was the best detected penicillin
5 673 for the penicillin family because penicillin G (LOD 5 $\mu\text{g l}^{-1}$) was the best detected penicillin
6 674 during the validation of the STAR protocol in milk and cloxacillin (LOD 60 $\mu\text{g l}^{-1}$) was the least
7 detected penicillin (Gaudin et al. 2004). Moreover they belong to 2 groups of MRL (penicillin
8 675 G 50 $\mu\text{g kg}^{-1}$ in muscle and cloxacillin 300 $\mu\text{g kg}^{-1}$). Similarly, erythromycin (LOD 30 $\mu\text{g l}^{-1}$)
9 676 and tilmicosin (LOD 50 $\mu\text{g l}^{-1}$) were the best detected macrolides in milk. On the contrary,
10 677 tylosin (LOD 200 $\mu\text{g l}^{-1}$) was the least detected and was representative of the group of
11 678 spiramycin (LOD 300 $\mu\text{g l}^{-1}$) and neospiramycin (LOD 200 $\mu\text{g l}^{-1}$). Moreover they also belongs
12 679 to 2 groups of MRL (tylosin 100 $\mu\text{g kg}^{-1}$ and erythromycin 200 $\mu\text{g kg}^{-1}$). Concerning
13 680 quinolones, enrofloxacin was considered as representative of the quinolones family, except
14 681 flumequine. In fact, flumequine is usually badly detected by many microbiological methods.
15 682 When the STAR protocol was validated in milk, we have observed that enrofloxacin
16 683 (detection limit = 20 $\mu\text{g l}^{-1}$), ciprofloxacin (10 $\mu\text{g l}^{-1}$), marbofloxacin (30 $\mu\text{g l}^{-1}$) and
17 684 danofloxacin (15 $\mu\text{g l}^{-1}$) had the same activity profiles, with similar sensitivities (Gaudin et al.
18 685 2004). The quinolones which was badly detected was flumequine (> 600 $\mu\text{g l}^{-1}$). During the
19 686 validation in milk, sulfanilamide, sulfapyridine, sulfadoxine, sulfacetamide, sulfaquanidine
20 687 were the least sensitive sulphonamides (3.5 to 20 times the MRL (MRL = 100 $\mu\text{g/l}^{-1}$) and the
21 688 most sensitive sulphonamides were sulfaphenazole, sulfathiazole, sulfachloropyridazine
22 689 (MRL). The limits of detection of sulfadimethoxine (SDMX) (1.75 times the MRL) and
23 690 sulfamethazine (2.5 times the MRL) were in between. The choice of sulphonamide was not
24 691 based on the least detected compounds because it was too far from the MRL but was
25 692 focused on 2 antibiotics which had intermediary sensitivities. Moreover these 2
26 693 sulphonamides are commonly used for animal treatment. Concerning the aminoglycosides,
27 694 gentamicin in milk was the best detected antibiotic (limit of detection 300 $\mu\text{g l}^{-1}$) and
28 695 spectinomycin was the least detected aminoglycoside (20000 to 40000 $\mu\text{g l}^{-1}$). The limit of
29 696 detection for neomycin, kanamycin, streptomycin and DHS was equal to 1000 $\mu\text{g l}^{-1}$.
30 697 Therefore DHS is representative of the 3 other aminoglycosides. Lincomycin was detected at
31 698 350 $\mu\text{g l}^{-1}$ and pirlimycin at 100 $\mu\text{g l}^{-1}$. Therefore lincomycin was the least detected and is
32 699 representative of the detection of lincosamides.
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34 701
35 702 The specificity of the STAR protocol for the detection of antimicrobial residues in porcine
36 703 muscle was very satisfactory. A similar result was obtained by Pikkemaat et al. (2009a) when
37 704 implementing the STAR protocol on routine monitoring muscle samples in a comparative
38 705 study. Only 1 % of false positive results were observed (6/591), on plate Bst, after the
39 706 analyses of 591 routine monitoring samples, when the cut-off was set at 4 mm, which is the
40 707 recommended cut-off in the STAR protocol for the plate Bst.

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3 708 The results were obviously similar to the global orientation given in the STAR protocol: beta-
4 lactams and sulphonamides were detected preferentially on plate Bst, tetracyclines on plate
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6 Bc6, quinolones on plate Ec8 and macrolides on plate Kv8. However, there were 2
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8 exceptions. The aminoglycosides have been detected on plate Bst with the concentrations
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10 tested for the determination of CC β , whereas for higher concentrations than CC β , they were
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12 detected on plate Bs8 (= Bs7.2). However, when the validation of the STAR protocol has
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14 been performed in milk, Bs8 was the specific plate for the detection of aminoglycosides.
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16 Secondly, tylosin was detected preferentially on Bst instead of Kv8 at this concentration,
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18 which had already been observed during the validation of the STAR protocol in milk. So if a
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20 positive result is reported on plate Bst, the confirmation should be directed onto beta-
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22 lactams, sulphonamides, but also tylosin and aminoglycosides. A comparative study of 3
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24 microbial screening tests including the STAR protocol applied to routine monitoring samples
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26 (Pikkemaat et al. 2009a) has similarly, shown that a macrolide (tulathromycin) was
27 720
28 preferentially detected on plate Bc6 and Bst instead of plate Kv8. Therefore if no
29 721
30 tetracyclines could be confirmed in a positive sample on plate Bc6, the confirmation could be
31 722
32 directed towards tulathromycin. During this comparative study, the STAR protocol was able
33 723
34 to detect the 4 MRL samples which contained antibiotic concentrations higher than their
35 724
36 respective MRLs. Three of them were tetracyclines, detected preferentially on plate Bc6 and
37 725
38 the remaining residue was sulfadiazine at 172 $\mu\text{g kg}^{-1}$. No information were available for
39 726
40 beta-lactams, macrolides and quinolones because none of these families were found in the
41 727
42 routine monitoring samples. The NAT-screening test was able to detect 4 samples containing
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44 aminoglycosides, but not the STAR protocol because this method is not enough sensitive
45 729
46 towards the aminoglycoside family. Furthermore, the NAT-screening test is applied to kidney,
47 730
48 while the STAR protocol is recommended for muscle. Moreover, it is well known that
49 731
50 aminoglycosides concentrated in kidney, while very low concentrations could be found in
51 732
52 muscle. The muscle matrix is not a satisfactory matrix for the screening of aminoglycosides.
53 733
54 The results of this comparative study are in accordance with our validation data.
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736 The detection capabilities have been determined for 16 antibiotics from different families in
737 relation to their respective Maximum Residue Limit (MRL). The levels of detection of beta-
738 lactams (penicillins and cephalosporins \leq MRL) were very satisfactory because even the
739 least detected compound has been detected at or below MRL. Concerning tetracyclines,
740 OTC (the least detected) could be detected at levels below 2.5 times the MRL and
741 doxycycline (the best detected) at the MRL. The levels of detection of macrolides (2 MRL),
742 quinolones (\leq 2 MRL) and trimethoprim (2 MRL) were also satisfactory even for the least
743 detected compound. The levels of detection of one representative sulphonamide SDMX (\leq 3
744 MRL) were slightly higher than in milk, Finally, the sensitivity of the STAR protocol towards

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3 745 aminoglycosides and florfenicol was not satisfactory (>>MRL). Therefore the interest of the
4 746 plate Bs8 in the STAR protocol could be discussed. This plate should be improved for its
5 747 sensitivity or should be replaced by another plate. Other possibility is to use a
6 748 complementary method focused on aminoglycosides which could replace the use of this fifth
7 749 plate.
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12 751 The validation of microbiological plate test is also a complex issue because of the solid
13 752 matrices like muscle and kidney. In fact, the ideal solution would be to produce incurred
14 753 samples from animal treatment for all antibiotics having MRL in the corresponding matrix and
15 754 to validate all these antibiotics. However this is extremely time consuming and expensive.
16 755 Furthermore, it is really difficult to obtain exactly a target concentration after the treatment of
17 756 animals. So there are different possibilities to implement the validation of a microbiological
18 757 plate test.
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27 759 The first option is to use spiked paper discs to determine the sensitivity of the method from
28 760 antibiotic standard solutions (Koenen-Dierick et al. 1995; Calderon et al. 1996, Currie et al.
29 761 1998; Ferrini et al. 2006). The main advantages are that this solution is less expensive and
30 762 quicker. The validation could be implemented for a wide list of antibiotic residues. However,
31 763 matrix components could affect the detection capabilities of a method. Okerman et al.
32 764 (1998a) showed that only tetracyclines and quinolones were similarly detected with or
33 765 without tissue. However, the detection of beta-lactams was better with antibiotic spiked discs
34 766 without tissue than with tissues. For some antibiotics, the difference was only observed at
35 767 low concentrations. Therefore, the use of antibiotic spiked discs is not totally satisfactory.

36 768 The second possibility is to use monitoring routine samples (Okerman et al. 1998b;
37 769 Pikkemaat et al. 2009a; Schneider et al. 2009). Routine samples from monitoring plans have
38 770 been analyzed with different methods including the method to be validated (including one or
39 771 several physico-chemical confirmatory methods). The first disadvantage is that the number of
40 772 samples containing residues is unknown. Moreover, very powerful confirmatory methods are
41 773 needed to confirm all the samples, preferably even negative results at the screening step to
42 774 check the false negative rate of the screening test. Therefore it is also costly and time
43 775 consuming. Finally only a narrow range of antibiotics (those which are most frequently used
44 776 in the country) is encountered. Schneider et al. (2009) confirmed the presence of antibiotics
45 777 in 29 samples, from which 23 belonged to the tetracycline family. This kind of validation led
46 778 very interesting information on the method, its practicability in routine conditions and some
47 779 information on its performance but the results are limited by the range of antibiotics and their
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3 781 Sometimes, the validation study combined spiked discs and analyses of incurred materials,
4 782 resulting from animal treatment (Myllyniemi et al. 1999; Myllyniemi et al. 2001). In this case,
5 783 the authors used the activity patterns of antibiotic standard solutions to identify antibiotic
6 784 residues in incurred materials. Furthermore, the STAR protocol has been validated in our lab
7 785 using spiked paper discs, plus analysing field routine samples. The interest of spiked discs is
8 786 that many antibiotics at many concentrations could be tested but limitation is that no matrix
9 787 effect could be observed. It is interesting to obtain preliminary data on the evaluation of a
10 788 new method and activity profiles of many antibiotics. But we know that matrix will affect the
11 789 detection capabilities of the method. Therefore, field samples are very interesting because
12 790 they are incurred materials and matrix effect could be demonstrated. However the limitation
13 791 is that it is not known in advance how many positive samples are in the panel of field
14 792 samples, which antibiotics would be detected and at which concentrations. Moreover another
15 793 limitation is that a little variety of antibiotic residues or families are usually found, depending
16 794 on the matrix. Okerman et al. (2004) used artificially contaminated (spiked tissue fluid) as
17 795 well as incurred samples for the comparative study of 4 screening methods for the detection
18 796 of tetracyclines in muscle matrix. This approach is also interesting because matrix effect
19 797 could be observed both with spiked tissue fluid and incurred tissue.

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32 799 The third possibility is the use of “simulated tissues” as it is proposed in this paper. It seemed
33 800 to be a good compromise between spiked discs and incurred tissues and the nearest
34 801 preparation from intact muscles because matrix effect could be evaluated. For the validation
35 802 of a microbial screening assay, Pikkemaat et al. (2007) used 2 different approaches to
36 803 produce fortified tissues. Firstly spiked minced was heated and centrifuged to extract meat
37 804 juice (supernatant) which was applied directly onto the plate. Secondly incurred tissues were
38 805 transformed into powder (using liquid nitrogen and blending the meat) and meat juice was
39 806 extracted. Therefore, it was possible to obtain accurate concentrations in the samples by
40 807 mixing incurred and blank materials. One conclusion was that “the sensitivity of the assay for
41 808 quinolones decreased 2 fold when matrix samples are analysed” by comparison with
42 809 antibiotic standard solutions. In these conditions, matrix effect was taken into account.
43 810 Moreover, these 2 approaches are fitted for this screening test because in routine, meat juice
44 811 samples would be analysed also. However, that sample preparation differed a lot from the
45 812 routine application of the STAR protocol which is based on pieces of raw muscles laid
46 813 directly on the plates. Therefore, in our case, our “simulated tissues” appeared to be as near
47 814 as possible from real muscle samples. The binding of antibiotics to the minced tissue is
48 815 possible during spiking. In 2009, Pikkemaat et al validated the NAT-post-screening test for
49 816 the detection of antibiotic residues in kidney by determining the detection capabilities of 36
50 817 antibiotics in porcine as well as in homogenised fortified kidney samples (Pikkemaat et al.

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3 818 2009b). After centrifugation, the supernatant could was analysed. The same final treatment
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5 819 was applied to routine monitoring samples. This is another way of preparing simulated
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7 820 tissues.

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10 822 In the decision EC/2002/657 (EC 2002), there is no recommendation concerning the kind of
11 823 materials to be analysed (spiked or incurred matrix) during the validation. Therefore all of the
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13 824 previous validation studies could be in accordance with the European decision if the way of
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15 825 determining performance characteristics was respected. The most important thing is to keep
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17 826 as close as possible to the materials analysed in routine use by the concerned method. Now
18 827 the guideline for validation of screening methods (Anonymous 2010) recommends the use of
19 828 "simulated tissues" (spiked tissues) when it concerns solid matrices and when it is impossible
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21 829 to obtain incurred tissues. Moreover, the validation could be restricted to a list of
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23 830 representative antibiotics. The validation which have been performed in the past on field
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25 831 samples led very interesting information concerning the method. However this kind of
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27 832 validation does not fit to the decision EC/2002/657 and to the recommendations of the
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29 833 validation guideline (Anonymous 2010). In fact the number of antibiotics tested is always very
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31 834 restricted. Moreover the number of samples to be analysed to determine the detection
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33 835 capabilities of antibiotics is never respected.

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36 837 As a conclusion, there is no ideal way of validating microbiological plate tests which would
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38 838 not be time and money consuming. Obviously, validation of these kind of methods needs a
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40 839 lot of efforts from the lab which would validate the protocol for the first time (initial validation).
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42 840 In the guideline for validation of screening methods (Anonymous 2010), one of the
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44 841 recommendations is to reduce the work of validation when the method is transferred to
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46 842 another laboratory which would want to implement it in routine conditions. In fact, the number
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48 843 of samples to be analysed is reduced. The performance characteristics (e.g. detection
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50 844 capabilities) determined in the transfer lab have to be compared to those determined during
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52 845 the initial validation for a selected range of antibiotics. Then the participation to proficiency
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54 846 testing studies could complete the validation dossier.

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57 848 **Conclusions**
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59 849 The two objectives of this validation study have been fulfilled. Firstly, the STAR protocol has
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850 been validated for muscle by determining performance characteristics (specificity, detection
851 capabilities CC β for 16 representative antimicrobials, applicability, ruggedness), according to
852 the European decision 2002/657/EC (EC 2002). The STAR protocol is applicable to the
853 broad spectrum detection of antibiotic residues in muscles of different animal species (pig,
854 cattle, sheep, poultry.). The method has good specificity (false positive rate 4%). The

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3 855 detection capabilities have been determined for 16 antibiotics from different families in
4 856 relation to their respective Maximum Residue Limit (MRL): beta-lactams (penicillins and
5 857 cephalosporins \leq MRL), tetracyclines (\leq MRL and \leq 2.5 MRL), macrolides (2 MRL),
6 858 quinolones (\leq 2 MRL), some sulphonamides (\leq 3 MRL), trimethoprim (2 MRL). However, the
7 859 sensitivity of the STAR protocol towards aminoglycosides and florfenicol was not satisfactory
8 860 (\gg MRL). Finally, this study has shown that the STAR method is a robust screening method,
9 861 insensitive to reasonable variations analytical parameters such as concentration of bacteria,
10 862 amount of medium, incubation period and pre-incubation or not.
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12 864 Secondly, the applicability of the European guideline (Anonymous 2010) to the validation of a
13 865 microbiological screening method and for muscle has been demonstrated. The use of
14 866 "simulated tissue" appeared to be a very good compromise between antibiotic spiked discs
15 867 and incurred samples. Moreover, the choice of a list of representative antimicrobials was also
16 868 very interesting to reduce the scope of validation.
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3 948 **Figure 1. Preparation of the simulated tissues.**

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5 949 *Two technicians prepared each 30 samples per antibiotic (AB), divided in 2 sets of 15*
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7 950 *samples each (Set 1 and Set 2). The 2 sets have been spaced out, to introduce variability in*
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9 951 *the period of analysis (ambient conditions) and therefore variability of batches (media,*
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11 952 *bacteria, antibiotic standard...). The example of preparation of Set 1 by technician 1 is*
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13 953 *presented here. Four different batches of porcine muscle have been used. Two different*
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15 954 *working solutions (WS) have been prepared from the stock solution of antibiotic and used to*
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17 955 *spike the different batches of muscle. Finally 15 samples have been prepared per antibiotic.*

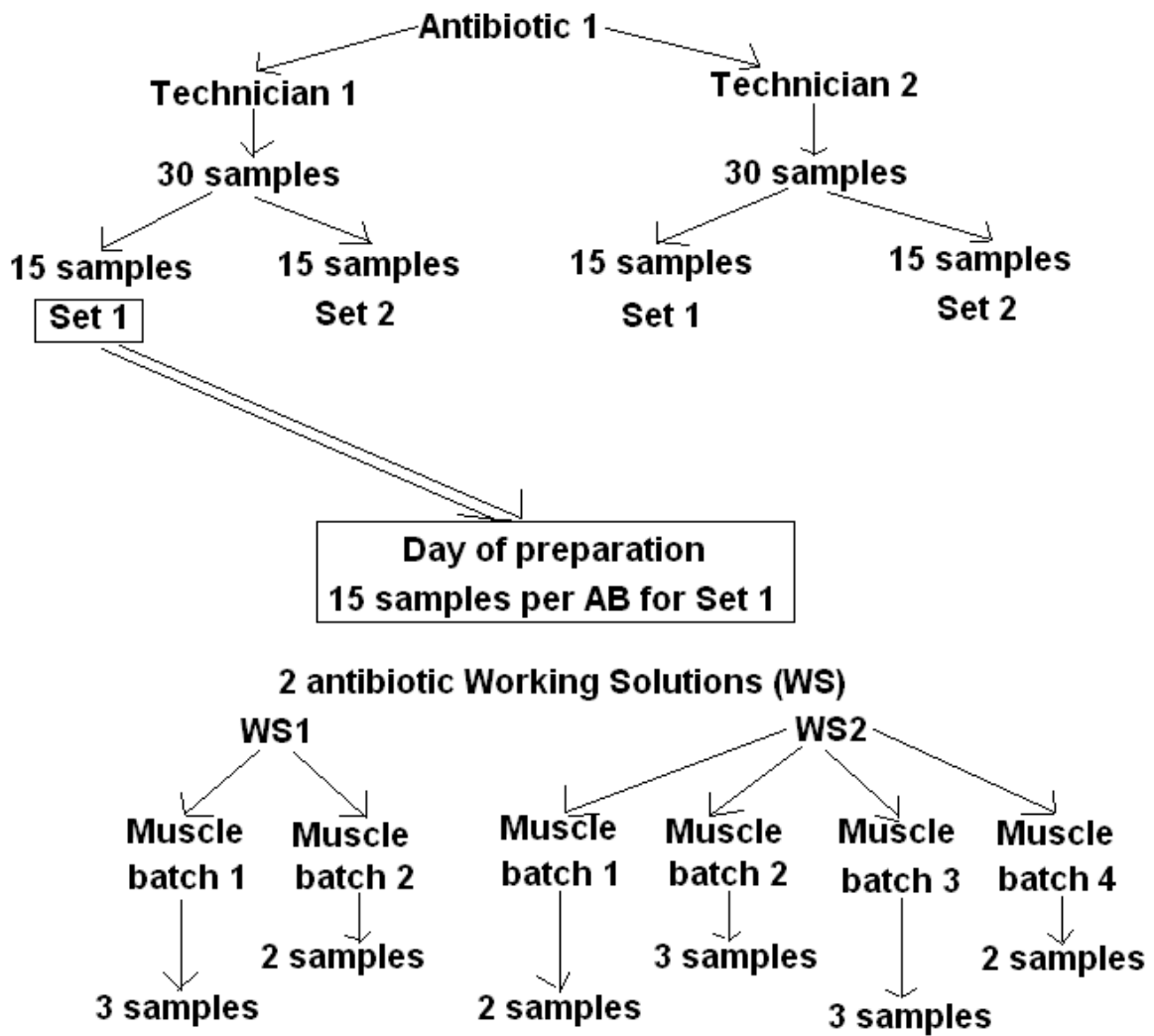
18 957
19 958 **Figure 2. Relationship between the calculated detection capabilities and the respective**
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21 959 **MRLs of the 16 representative antibiotics.**

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23 960 *The 16 representative antibiotics are represented in x-axis. The calculated $CC\beta$ of the 16*
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25 961 *tested representative antimicrobials and the respective MRLs are represented in y-axis. MRL*
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27 962 *= Maximum Residue Limit.*

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31 965 **Figure 3. Results of the applicability study of the STAR protocol to muscles from**
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33 966 **different animal species, on the 5 different plates.**

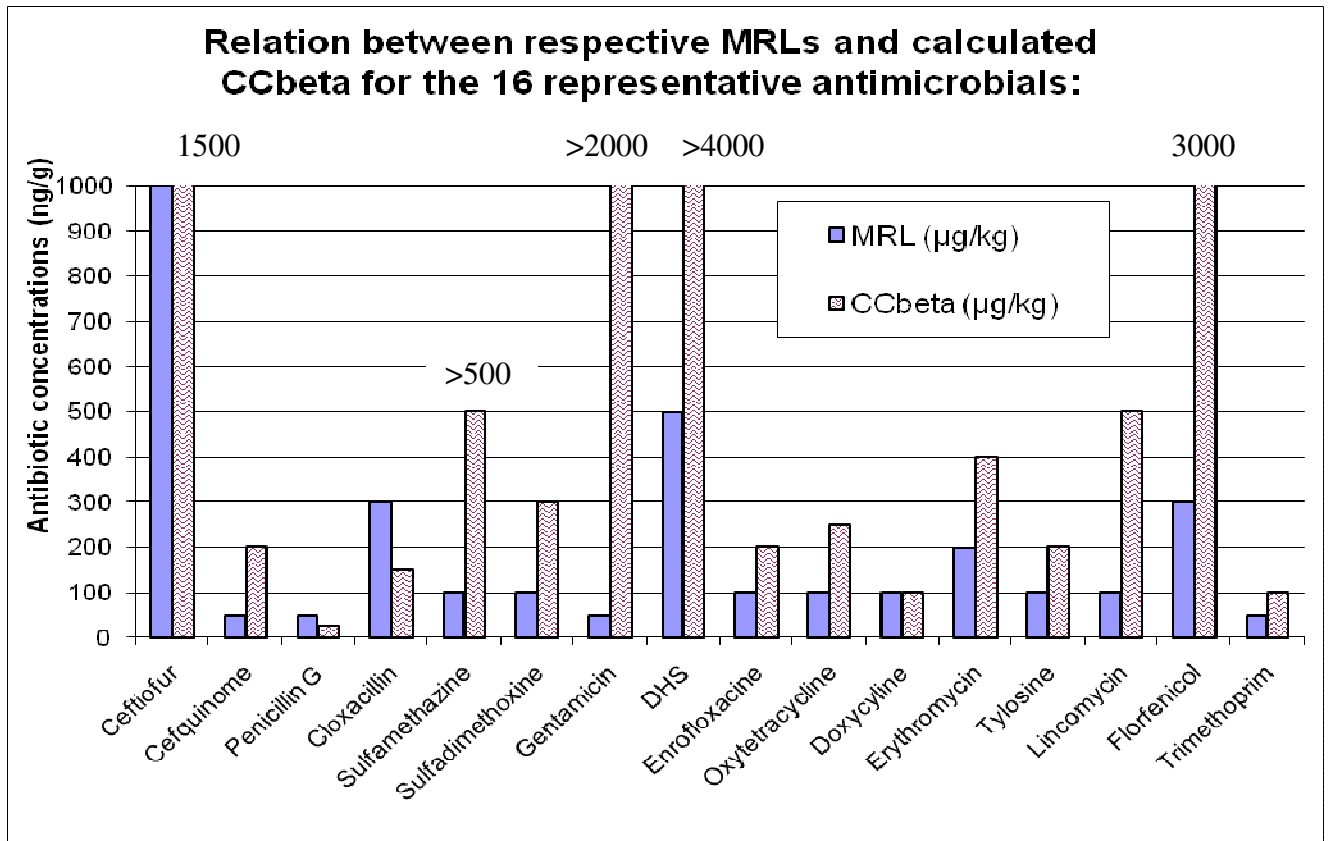
34 967 *The results of the applicability study for the 5 plates are represented: 2a. penicillin G at 25 μg*
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36 968 *kg^{-1} on plate Bst, 2b. doxycycline at 100 μg kg^{-1} on Plate Bc6, 2c. erythromycin at 400 μg kg^{-1}*
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38 969 *on Plate Kv8, 2d. enrofloxacin of 200 μg kg^{-1} on Plate Ec8 and 2e. gentamicin at 6000 μg kg^{-1}*
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40 970 *on Plate Bs8. In x-axis, the 4 different animal species tested are represented. In y-axis, the*
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42 971 *average inhibition zone (in mm) on the 5 different plates obtained during the applicability*
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44 972 *study for bovine, ovine and poultry muscles and during the determination of $CC\beta$ for porcine*
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46 973 *muscle are represented for blank samples and spiked samples.*

Figure 1.



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Figure 2.



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Figure 3.

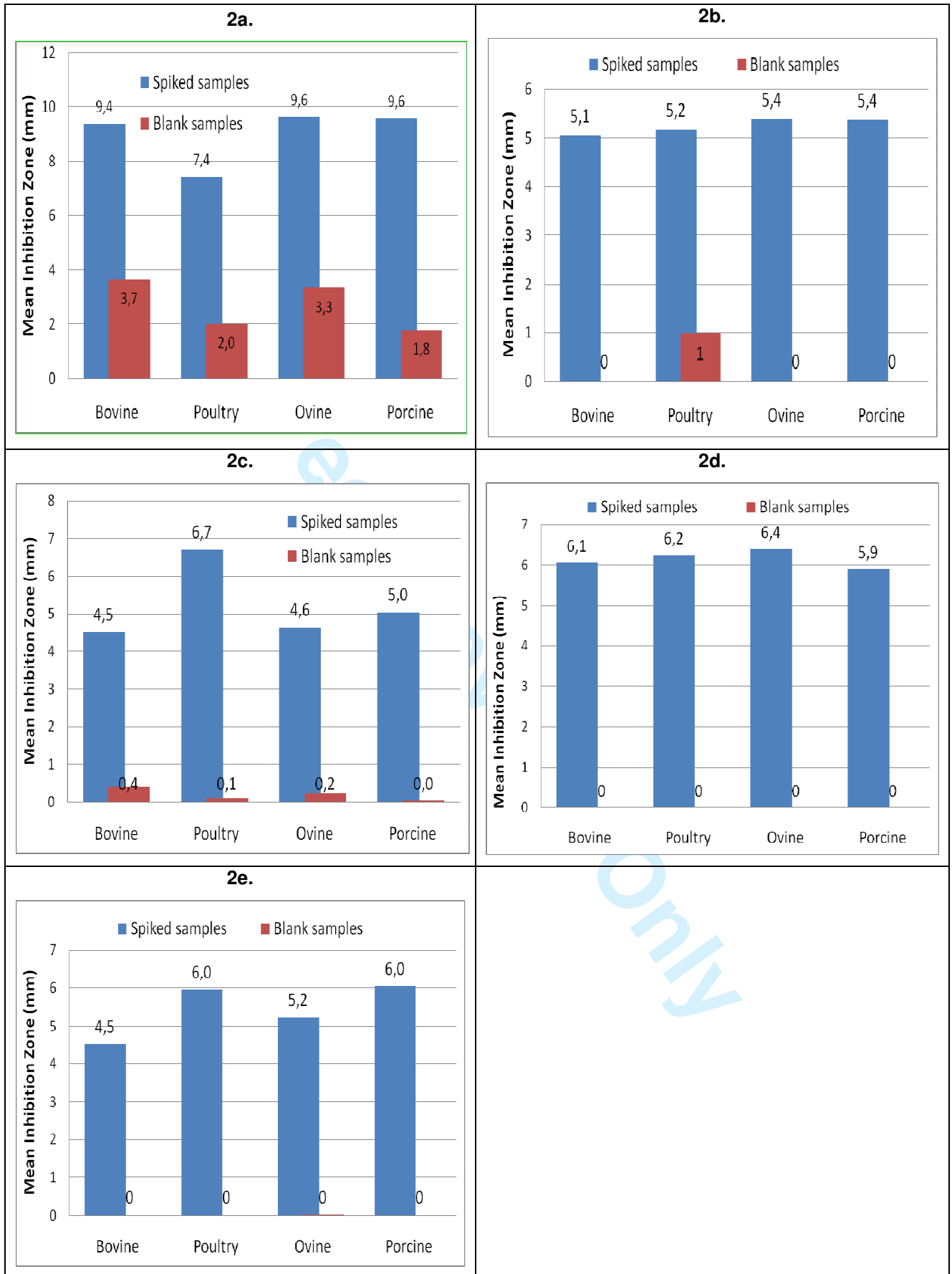


Table 1. Chosen antibiotics and corresponding concentrations for the validation study.

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

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

Table 2. Design matrix of the ruggedness study .

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

Run 1 = Day 1

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

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

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

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

Table 3. Detection capabilities $CC\beta$ ($\mu\text{g kg}^{-1}$) for the 16 antimicrobials

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

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

Table 4. Specificity of the plates.

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

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

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

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

SD = Standard Deviation

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

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

^a Concentration of bacteria; ^bMedium quantity; ^c Incubation time, ^d Pre-incubation time

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

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

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

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

Run 1 = Day 1

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