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Valerie Gaudin, Celine Hedou, Annie Rault, Pascal Sanders, Eric Verdon

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Comparative study of 3 screening tests, ExplorerÒ test, PremiÒTest2 microbiological tube tests and a multi-sulphonamides ELISA kit, for the detection of antimicrobial and sulphonamide residues in eggs

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2 1 **Comparative study of three screening tests, two microbiological tube**
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5 2 **tests and a multi-sulphonamide ELISA kit, for the detection of**
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8 3 **antimicrobial and sulphonamide residues in eggs**
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14 6 V. GAUDIN*, C. HEDOU, A. RAULT, P. SANDERS & E. VERDON
15

16 7 *Community Reference Laboratory for Antimicrobial Residues, AFSSA Fougères – LERMVD, La*
17

18 8 *Haute Marche – BP 90203, 35302 FOUGERES Cedex, France*
19
20
21 9

22
23 10
24
25 11
26
27 12 *To whom correspondence should be addressed.
28

29 13 e-mail: v.gaudin@fougeres.afssa.fr
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Abstract

The screening of antimicrobial residues in eggs is especially an important subject. Three different commercial kits for the screening of sulphonamides and other antimicrobials in eggs have been validated in accordance with the decision 2002/657/EC (EC 2002): one ELISA kit multi-sulphonamides (from RAISIO Diagnostics) and 2 microbiological tests (Premi®Test from DSM and Explorer® kit from Zeu-Inmunotec). The false positive rates were lower than 2 % for all kits. The detection capabilities ($CC\beta$) have to be as low as possible for banned substances and lower than the MRL when MRLs have been set. The sensitivity of the Premi®Test was better than that of Explorer® test, probably because of the dilution of the eggs before Explorer® test. The $CC\beta$ values towards most of the tested sulphonamides were satisfactory with Premi®Test ($\leq 100 \mu\text{g kg}^{-1}$). The performance in a proficiency test for the detection of sulphonamides in eggs with Premi®Test confirmed these results. The detection capability of tetracycline and doxycycline were at the level of the MRL or twice the MRL maximum. The detection capabilities for chlortetracycline and oxytetracycline were higher (4 to 6 times the MRL). The detection capabilities for amoxicillin, neomycin, tylosin and erythromycin were lower than their respective MRLs. Detection capabilities for sulphonamides were much lower for ELISA kit than for microbiological tests. The ELISA kit (from RAISIO Diagnostics) could be recommended for the targeted screening of sulphonamides in eggs. On the other hand, Explorer® test and Premi®Test could be used as wide screening tests allowing to detection of most of the antimicrobial families.

Keywords: *Validation, inhibition test, ELISA test, screening assay, egg samples.*

39 Introduction

40 Eggs are a staple food in the world for many populations providing a vital sources of animal
41 protein. Antimicrobials are used as feed additives or in drinking water in poultry breeding to prevent
42 animal diseases (enteric diseases) (Dahiya et al. 2006) or as growth promoting factors (Donoghue
43 2003; Stolker et al. 2007). The use of antimicrobials in laying hens could result in the persistence
44 of residues in eggs. Different studies of the excretion of tetracyclines and their epimers have been
45 performed after oral medication of laying hens (Roudaut et al. 1989; Zurhelle et al. 2000; Donkova
46 et al. 2005), macrolides (Roudaut et al. 1990); sulphonamides (Shaikh et al. 2000; Roudaut 2002;
47 Shaikh et al. 2004), ampicillin (Donoghue et al. 1997). The conclusion was that residues of
48 antimicrobials could persist in eggs several days after medication in drinking water. Furthermore,
49 Kan (2000) reviewed several studies on drug residues in eggs and their distribution between yolk
50 and white, after administration to laying hens. Some models were established to evaluate the
51 persistence of antimicrobial residues in eggs. A sulfamethazine model was presented by Tansakul
52 (2007) which demonstrated that even low doses of sulfamethazine could result in the presence of
53 residues in eggs and therefore could present a risk for consumer safety. As a conclusion, nearly all
54 veterinary drugs and feed additives available on the market may result in residues in eggs (Kan et
55 al. 2007).

56
57 Therefore, the detection of antimicrobial residues in eggs is especially of great interest both for its
58 impact on public health. Moreover, the presence of residues could lead to bacterial resistance to
59 different antimicrobials (i.e. streptomycin and tetracycline) (Adesiyun et al. 2007). A study
60 conducted by the Canadian Food Inspection Agency (CFIA) reported the level of contamination of
61 eggs with antimicrobials (Quon 2000). This study was conducted over a period of 2 years on both
62 domestic and imported eggs. More than 99% of the samples screened were found to be free of any
63 veterinary drug residue. The other samples contained tetracyclines, sulphonamides, ciprofloxacin,
64 macrolides, and streptomycin. Another study conducted in Trinidad concluded that the
65 antimicrobial residues detected in table eggs were particularly sulphonamides and then
66 macrolides, tetracyclines, and beta-lactam (Adesiyun et al. 2005). Sulphonamides are still effective
67 tools in the treatment of coccidiosis in broiler chicken and are widely used in intensive poultry

1
2 68 farms. The Committee for Veterinary Medicinal Products recommended that the sulphonamides
3
4 69 are entered into Annex I of Council Regulation (EEC) 2377/90 (EC 1990). The MRL of
5
6 70 sulphonamides for meat and milk is 100 µg kg⁻¹. However, there is no MRL set for sulphonamide in
7
8
9 71 egg products. In fact, there are no sulphonamides and no beta-lactams approved for use in laying
10
11 72 hens. Among macrolides, tylosin is intended to be administered to laying hens, in the drinking
12
13 73 water or in premix formulations, for the treatment of respiratory diseases and necrotic enteritis.
14
15 74 Erythromycin is used for the treatment of chronic respiratory diseases due to mycoplasma in
16
17 75 poultry. In veterinary medicine, tetracyclines are allowed for medication of laying hens and used as
18
19 76 a broad spectrum antibiotic. Neomycin is used to treat bacterial gastrointestinal infections of poultry
20
21 77 by the oral route. The previously cited antimicrobials are authorised to be administered to laying
22
23 78 hens. The European Union has identified Maximum Residue Limits (MRL) for some molecules but
24
25 79 very few antimicrobials have MRL in eggs (Table I).

26
27
28 80 Insert Table I here.

29
30 81 When the use of some antimicrobials is forbidden for all animal species (i.e. chloramphenicol), they
31
32 82 are entered in Annex IV of the Regulation EC 2377/90 (EC 1990). Furthermore, some
33
34 83 antimicrobials are forbidden to be administered to laying hens produced for human consumption
35
36 84 only (i.e. doxycycline, spectinomycin, enrofloxacin). However, other antimicrobials (i.e. penicillins,
37
38 85 sulphonamides) have no MRL set for eggs (thus a “zero” tolerance). Therefore, in both cases, the
39
40 86 detection capabilities of the methods have to be as low as possible as a consequence of no
41
42 87 authorisation of these antimicrobials in eggs and no MRL set for this product.
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45 88

46
47 89 Due to the possible persistence of antimicrobial residues in eggs, the presence of antibiotics must
48
49 90 be controlled with relevant analytical methods to meet the EU regulations. There are very few
50
51 91 publications at this time describing screening methods for the detection of antimicrobial in eggs.
52
53 92 However, the screening step is the first step of the control and thus is an essential step. There is a
54
55 93 need of wide spectrum screening tests, quick, easy to use and cheap. Different screening tests
56
57 94 were developed for the screening of antimicrobials in eggs: microbiological plate tests (Kabir et al.
58
59 95 2004; Bendix et al. 2005; Pikkemaat et al. 2007), tube tests like Premi®Test (Hussein et al. 2005)
60
96 and Explorer® test, enzyme linked immunosorbent assay (ELISA) (Huet et al. 2006), receptor test

1
2 97 (Lamar et al. 2007), dipstick-test for tetracyclines (Alfredsson et al. 2005), Charm II test ((Adesiyun
3
4 98 et al. 2005)) or physicochemical methods like HPLC (Horii et al. 1990; Herranz et al. 2007) and
5
6 99 LC/MS-MS methods (Wang et al. 2005; Heller et al. 2006; Stolker et al. 2007). To our knowledge,
7
8
9 100 there is no scientific publication at this time describing the use of Explorer® test for the screening
10
11 101 of antimicrobials in eggs.

12
13 102
14
15 103 An interlaboratory study was organised for the screening and the confirmation of different
16
17 104 sulphonamides in eggs by our laboratory as Community Reference Laboratory. On this occasion, a
18
19 105 study was conducted to evaluate the performances of 3 different commercial kits for their ability to
20
21 106 detect sulphonamides and other antimicrobials in eggs: one ELISA kit multi-sulphonamides (from
22
23 107 RAISIO Diagnostics, Diffchamb, Lyon, France) and 2 microbiological tests (Premi®Test from DSM,
24
25 108 Delft, The Netherlands and Explorer® kit from Zeu-Immunotec, Saragossa, Spain). Premi®Test
26
27 109 and Explorer® test are 2 commercial microbiological based tests. These kits, based on the
28
29 110 inhibition of bacterial growth (*Bacillus stearothermophilus* strain), contain a redox indicator that
30
31 111 changes colour in the absence of antibiotics in the sample. They are simple and fast methods for
32
33 112 the detection of antibiotics in fresh meat, eggs and feed samples. Technical guidelines and
34
35 113 performance criteria (e.g., detection capability $CC\beta$, selectivity and specificity) for residue control in
36
37 114 the framework of Directive 96/23/EC (EC 1996) are described in Commission Decision
38
39 115 2002/657/EC (EC 2002). This paper reports the validation of 3 kits for the detection of
40
41 116 sulphonamides and other antimicrobials in egg samples according to the Commission Decision
42
43 117 2002/657/EC (EC 2002).
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50 118 51 119 **Materials and methods**

52 53 120 *Explorer® test*

54
55
56 121 Explorer® test is a commercial kit for detection of inhibitory substances in raw meat and eggs. The
57
58 122 test, a 96-well microtiter plate, is based on the inhibition of microbial growth. Each well contains
59
60 123 specific agar medium containing a redox indicator and spread with *Geobacillus stearothermophilus*
124 spores.

1
2 125
3
4 126 *Preparation of egg samples.* Whole eggs (white and egg yolk) were homogenised and diluted (v: v
5
6 127 1:3) with distilled water (2 g egg + 6 ml distilled water). The diluted sample was heated for 12 min
7
8
9 128 at 100 ° C in a water bath and stirred vigorously 20-30 seconds to prevent clotting. The sample
10
11 129 was allowed to cool at room temperature.

12
13 130
14
15 131 *Test procedure.* 100 µl of diluted egg was added to each well. The wells were sealed with a plastic
16
17 132 film and the microplate was pre-incubated at 65°C for 30 min in a block heater (Zeu-Inmunotec,
18
19 133 Spain) and the sample allowed to diffuse through the well (pre-diffusion). Afterwards the wells were
20
21
22 134 washed by filling the wells up with distilled water, using a squeeze bottle. The washing step was
23
24 135 repeated 3 times to 4 times. The plate was emptied by turning the plate upside down on top of an
25
26 136 absorbent paper to remove the water in excess. NB: It should not remain traces of coagulated
27
28 137 egg!! The plate was sealed again with plastic wrap and incubated at 65°C until the negative control
29
30 138 sample has turned orange (approximately 3h-3h30min).

31
32 139
33
34 140 *Readings and interpretation of Explorer® results.*

35
36
37 141 The photometric reading of microplates is based on the reading at 2 wavelengths (590 and 650
38
39 142 nm) on a microplate reader. The results were interpreted as the difference between the values of
40
41 143 the two readings. On each day of validation, one negative control was analysed and also one
42
43 144 positive control (penicillin G at 25 µg kg⁻¹) (Quality Control QC). The plate is ready to be read when
44
45 145 the result for the negative control sample (difference of absorbance NA590nm - NA650 nm) is
46
47
48 146 between 0.15 and 0.25 OD (optical density units), where NA: Negative control absorbance. A
49
50 147 sample is declared positive when: SA 590nm - SA 650 nm ≥ NA590nm - NA 650nm + 0.15,
51
52 148 where : SA: Sample absorbance.

53
54 149
55
56 150 One single day of validation is presented as an example of interpretation. The result of each
57
58 151 unknown sample (SA = Sample Absorbance) is compared to the line of negative control sample
59
60 152 absorbance (NA) + 0.15. When: SA 590nm - SA 650 nm ≥ NA590nm - NA 650nm + 0.15, the

1
2 153 unknown sample was declared positive. An example of interpretation of quantitative results
3
4 154 (Optical Density) is presented on Figure 1.

5
6 155 Insert figure 1

7
8 156

9
10
11 157 *Premi® test*

12
13 158 Premi®Test is a commercial test for the screening of antimicrobial residues in fresh meat as well
14
15
16 159 as in fish and eggs. Premi®Test gives a result in less than four hours.

17
18 160 *Preparation of egg samples.* Egg sample was homogenised (white and yolk).

19
20 161 *Test procedure.* A syringe with a set volume of 100 µl is supplied with the kit. 100 µl of
21
22 162 homogenized egg fluid was transferred onto the agar in the ampoule, by pressing the syringe and
23
24 163 releasing it. The ampoule is closed with the plastic foil supplied with the kit. The ampoule(s) was
25
26 164 placed in a water bath at 80°C for 10 minutes. After this heat pre-treatment, the ampoules were
27
28
29 165 incubated at the required temperature of 64°C (± 0,5 °C). The ampoules were incubated until the
30
31 166 negative control (egg fluid what has been tested before negative) changed colour (turned yellow).

32
33 167 *Readings and interpretation of Premi®Test results.* When the negative control changes colour from
34
35 168 purple to yellow (approx. 3 hours), the results could be read from the bottom 2/3 part of the
36
37 169 ampoule. A clear colour change purple to yellow indicates that the antimicrobial compounds were
38
39
40 170 below the Premi®Test detection limits. A purple colour indicates the presence of antibiotics at or
41
42 171 above the detection limits of the Premi®Test.

43
44 172
45
46 173 *Multi-sulphonamides ELISA kit (RAISIO Diagnostics)*

47
48
49 174 This commercial multi-sulphonamide test is a competitive enzyme immunoassay (EIA) for
50
51 175 quantitative screening of tissue, milk, honey, egg and urine samples. The method should detect a
52
53 176 wide range of sulphonamides. It is based on monoclonal antibodies (21C7) raised against protein
54
55 177 bound sulfamethazine. The manufacturer claimed that it offers high sensitivity for sulfamethazine,
56
57 178 sulfadiazine, sulfamerazine, sulfisoxazole, sulfachloropyridazine and sulfachloropyrazine. This kit
58
59 179 is suitable for screening tissue, egg, milk, honey and urine for sulphonamides.

1
2 180 *Preparation of egg samples.* Egg sample was homogenised (white and yolk). 1 g was weighted
3
4 181 into a clean tube. 5 ml of ethyl acetate were added and thoroughly mixed with a vortex, followed by
5
6 182 mixing head over head for at least half an hour. After centrifugation (5 min., 2000xg), 1 ml of
7
8 183 supernatant (ethyl acetate) was pipetted into a clean tube and evaporated to dryness under a mild
9
10 184 stream of nitrogen at 50°C. The residue was dissolved in 1 ml of phosphate buffered saline (PBS*).
11
12 185 Then 1 ml of iso-octane/trichloromethane (2:3; v/v) was added and mixed using a vortex. After
13
14 186 centrifugation (5 min., 2000xg), 100 µl of supernatant was pipetted into a clean tube and 300 µl of
15
16 187 PBS* was added. Finally 50 µl portions were used for ELISA.

17
18
19 188 *PBS is not provided in the kit. The buffer is prepared as follows: Na₂HPO₄ 0.77 g, KH₂PO₄ 0.18
20
21 189 g, NaCl 8.94 g, distilled water qsp. 1000 ml. pH 7.4 ± 0.2

22
23
24 190 *Test procedure.* 100 µl of the zero standard in duplicate (wells A1, A2, blank), 50 µl of the zero
25
26 191 standard in duplicate (wells B1, B2, maximal signal), 50 µl of each of the sulfamethazine standard
27
28 192 solutions in duplicate (wells C1,2 to H1,2 i.e. 0.125, 0.25, 0.5, 0.8, 2.5 and 5 ng ml⁻¹) and 50 µl of
29
30 193 each extracted sample in duplicate were pipetted. Then 25 µl conjugate (sulphonamide-HRPO)
31
32 194 and 25 µl antibody solution were added to all wells, except wells A1 and A2. The microtiter plate
33
34 195 was sealed and shaken for a few seconds. The plate was incubated for 1 hour in the dark between
35
36 196 +2°C and +8°C). The microtiter plate was washed 3 times with rinsing buffer. Then 100 µl
37
38 197 substrate/chromogen solution was added to all wells. The plate was again incubated, 30 min at
39
40 198 room temperature in the dark. Finally the reaction was sopped by adding 100 µl of stop solution to
41
42 199 each well.
43
44

45 200 *Readings and interpretation of ELISA Test results.* Immediately after adding the stop solution, the
46
47 201 absorbance values at 450 nm was read. The values (% maximal absorbance) calculated for the
48
49 202 standards are plotted (on the Y-axis) versus the sulphonamide equivalent concentration (ng ml⁻¹)
50
51 203 on a logarithmic x-axis. The amount of sulphonamide in the samples is expressed as
52
53 204 sulphonamide equivalents. The sulphonamide equivalents in the extracts (ng ml⁻¹) corresponding
54
55 205 to the % maximal absorbance of each extract can be read from the calibration curve.
56
57

58 206
59
60 207 *Quality controls (QC)*

1
2 208 As it was strongly recommended by the manufacturer ((Premi®Test, DSM and Explorer®, Zeu-
3
4 209 Immunotec), a negative control sample (antibiotic-free egg) was used to determine the optimal
5
6 210 incubation time of each assay. Moreover, a positive sample (egg sample spiked with high
7
8 211 concentration of antibiotic, ie. Penicillin G 25 µg kg⁻¹) was tested at each day of validation to check
9
10
11 212 the performance of the kit.

13 14 213 *Validation*

15
16 214 *Specificity and false positive rate.* To determine these parameters, at least 20 egg samples from
17
18 215 different origins were analysed in blind duplicate for each of the 3 kits. Finally, even more than 20
19
20 216 blank egg samples were analysed with Premi®Test (33 blank egg samples) and with Explorer®
21
22 217 test (55 blank egg samples) (1 to 3 were analysed at each day of validation). However, only 12
23
24 218 blank samples have been analysed at the end with the ELISA kit. In practice, some experiments
25
26 219 failed because of stability issues of the conjugate. The instability of the conjugate was observed
27
28 220 when some days the optical density of the blank standard was lower than 0.6 OD, which was the
29
30 221 validity limit for the kit. For this reason, the number of negative samples that could be analysed
31
32 222 should be reduced to 12 samples only.

33
34 223
35
36 224 *Detection capabilities.* The concentration level, where only ≤ 5 % false compliant results remain,
37
38 225 equals the detection capability of the method (EC 2002).

39
40 226 A preliminary study allowed us to choose for all the 3 kits, the target concentrations for each
41
42 227 sulphonamide and each antimicrobial. It is the least detectable concentration in eggs. Then, at
43
44 228 least 20 egg samples were spiked for each sulphonamide and each antimicrobial, for at least one
45
46 229 concentration level (MRL or claimed detection capability). 20 analyses have to be carried out in
47
48 230 order to ensure a reliable basis for this determination. These samples were analysed in blind
49
50 231 duplicate with each test.

51 232
52
53 233 The RAISIO Diagnostics ELISA kit was a multi-sulphonamides ELISA kit which was not able to
54
55 234 detect other antimicrobial residues. Therefore only sulphonamides have been tested for their
56
57 235 detection by the ELISA kit.

1
2 236
3
4 237 *Selection of the antimicrobial compounds and concentrations.* The sulphonamides (sulfamethazine
5
6 238 (SMZ), sulfadiazine (SDZ), sulfamethoxazole, sulfadoxine, sulfamerazine, sulfadimethoxine,
7
8 239 sulfisoxazole, sulfachloropyridazine, sulfamethizole, sulfathiazol) and the tested concentrations
9
10 240 were selected by the CRL according to the data given by the 3 manufacturers on detection limits.
11
12 241 The other antimicrobials were chosen because some of them have MRL in eggs (chlortetracycline
13
14 242 (CTC), oxytetracycline (OTC), tetracycline (TTC), erythromycin, tylosin and neomycin). The 2
15
16 243 others (doxycycline, amoxicillin) were selected because they are from the main families of
17
18 244 antimicrobials (Table II).
19
20

21
22 245 Insert Table II here
23

24 246 For preparation of antibiotic stock solutions, antibiotic standards with known purity were used (from
25
26 247 Sigma Aldrich, France; Cluzeau InfoLabo, France). The stock solutions were diluted with distilled
27
28 248 water to prepare spiked samples.
29

30 249
31
32 250 *Cross-reactivities.* The percentages of cross-reactivities between SMZ and other sulphonamides
33
34 251 were only calculated for the multi-sulphonamides ELISA kit when determining the detection
35
36 252 capability CC_{β} . The cross-reactivity percentages (%) will be compared with those claimed by the
37
38 253 manufacturer.
39
40

41 254
42
43 255 *Precision.* The precision was determined as it is written in the European Decision EC/2002/657
44
45 256 (EC 2002) for the RAISIO Diagnostics ELISA kit which was the only quantitative test. In this
46
47 257 decision, the precision has to be determined for quantitative screening tests. For authorised
48
49 258 substances (MRL), the precision has to be determined at 0.5, 1 and 1.5 times the MRL. For
50
51 259 banned substances, the precision has to be determined at 1, 1.5 and 2 times the MRPL, where
52
53 260 MRPL is the Minimum Required Performance Limit. Concerning the detection of sulphonamides in
54
55 261 eggs, we have no MRL and no MRPL. In the literature, validation studies for sulphonamides in
56
57 262 eggs are commonly based on a target concentration of $100 \mu\text{g kg}^{-1}$ (Tarbin et al. 1999; Heller et al.
58
59 263 2006; Wang et al. 2007). Therefore, the evaluation of precision was performed with a target
60
264 concentration of $100 \mu\text{g kg}^{-1}$, as it was the MRL value at. Two samples of blank egg samples were

1
2 265 spiked with sulfamethazine solutions to yield concentrations equivalent to 0.5, 1 and 1.5 times the
3
4 266 MRL. At each level, the analysis at least six replicate analyses were performed each day of
5
6 267 analysis. These steps were repeated on 4 days. The SMZ concentration detected in each sample
7
8
9 268 was calculated. Finally, the mean concentration, standard deviation (SD) and the coefficient of
10
11 269 variation (CV %) of the fortified samples were calculated. Different experiment were performed:
12
13 270 Same batch, same technician (repeatability conditions) (2 days) and other batch, same technician
14
15 271 (reproducibility conditions) (2 days).

16
17 272
18
19 273 *Preparation of spiked egg samples.* Egg samples were spiked on each day of validation. 20 µl of
20
21 274 each antimicrobial solution was added to 2 g of egg sample. All the samples were codified to
22
23
24 275 perform blind analyses.

25
26 276
27
28
29 277 *Stability.* No experiments have been carried out to determine the stability of sulphonamides or
30
31 278 other antimicrobials in eggs. A stability study of different antibiotics in solution and in kidney juices
32
33 279 was already published (Cantwell et al. 2006). However, because the 3 tests could not distinguish
34
35 280 between all sulphonamides, the stability study should have been performed for all detected
36
37
38 281 sulphonamides in separate experiments. Furthermore, Premi® Test and Explorer® test could not
39
40 282 distinguish between all detected antibiotics. The number of experiments would have been high.
41
42 283 This kind of study would have become expensive and the workload would have been very high.
43
44 284 Finally, the ELISA kit (from RAISIO Diagnostics) allowed to report quantitative results. However,
45
46 285 our experience showed that ELISA kits and other immunological methods like BIACORE methods
47
48
49 286 (Gaudin et al. 2007) are not fitted to test stability of drugs because of their high variability.
50
51 287 Therefore, the stability of the analytes in the egg matrix was studied by a bibliographical study.

52
53 288
54
55 289 *Participation to a proficiency test*
56
57
58 290 A proficiency test (PT) was organised by the Community Reference Laboratory (CRL) in Fougères
59
60 291 to evaluate the participants' methods to detect, confirm and quantify residues of sulphonamides in
292 whole eggs. The participants were free to use the method of their choice. Therefore several

1
2 293 different methods have been implemented (in house methods or commercial kits). The participants
3
4 294 were National Reference Laboratories (NRLs) from Member States and Third Countries. As the
5
6 295 French NRL, our laboratory has participated to the PT with the Premi@Test. However, the
7
8 296 technicians who implemented the Premi@Test were not involved in the organisation of the PT and
9
10
11 297 did not know the content of samples. Six samples were sent to the participants in December 2006,
12
13 298 2 blank samples and 3 spiked samples (materials 3, 4, 5). 2 blank samples were sent (1 laying
14
15 299 hens (material 1) and 1 quail egg (material 2)). Material 3 contained sulfadiazine (SDZ) at 50 μg
16
17 300 kg^{-1} and sulfadimethoxine (SDMX) at 60 μg kg^{-1} , material 4 contained sulfamethazine (SMZ) at 50
18
19 301 μg kg^{-1} and material 5 contained sulfadiazine at 30 μg kg^{-1} . Material 4 (a/b) was sent in blind
20
21 302 duplicate.
22
23
24 303
25
26 304
27
28 305
29

30 306 **Results and discussion**

31 32 33 307 *Specificity and false positive rate*

34
35 308 *Explorer® test.* Only one false positive result was obtained out of 55 blank egg samples.
36
37
38 309 Therefore, the false positive rate was satisfactory (1.82%). 2 different batches of Explorer® were
39
40 310 used during this study. Moreover, 2 different batches of eggs were also analysed.
41
42 311
43

44 312 *Premi@Test.* 33 blank egg samples have been tested finally. The false positive rate was 0%.
45
46 313
47

48
49 314 *ELISA kit (RAISIO Diagnostics).* For the 12 blank egg samples, calculated SMZ concentrations
50
51 315 were very low (between 0.0 +/- 0.0 and 4.5 +/- 1.9 μg kg^{-1} over the 6 days). The overall mean was
52
53 316 2.2 +/- 2.0 μg kg^{-1} . The distribution of the SMZ concentrations of the 12 blank samples was
54
55 317 represented on figure 1.
56

57 318  Insert Figure 1
58

59 319 The difference of calculated SMZ concentrations between blank samples and spiked SMZ samples
60
320 at 25 μg kg^{-1} is clearly observable. No false positive results were observed.

1
2 321 The pre-incubation at 80°C during 10 minutes with Premi®Test and during 12 minutes from
3
4 322 Explorer® test allowed to reduce the false positive rate due to the possible presence of naturally
5
6 323 inhibitors in eggs. The extraction prior the ELISA test also contributed to reduce the false positive
7
8 324 rate.

9
10
11 325
12
13 326 *Detection capabilities.*

14
15 327 The detection capabilities were calculated with one tested concentration for each test. Therefore, if
16
17
18 328 at least 95% of the spiked samples were found positive with the test, the detection capability was
19
20 329 lower or equal to the tested concentration. If less than 95 % of the spiked samples were found
21
22 330 positive with the test, the detection capability was higher to the tested concentration.

23
24 331
25
26 332 *Sulphonamides.* The CC_β values obtained for the 3 kits for sulphonamides are compared in Table

27
28
29 333 III.

30
31 334 Insert Table III here

32
33 335 *Explorer® Test.*

34
35
36 336 Sensitivity of Explorer® test was calculated in eggs by the manufacturer according to the EN ISO
37
38 337 13969:2003 regulation. Samples without any antibiotics or sulphonamides were spiked with
39
40 338 different concentrations of antimicrobial compounds, and at least eight replicates of every
41
42 339 concentration were analysed on different days. The concentration of antibiotic producing 95%
43
44 340 positive samples was considered as the limit of detection for that antibiotic. Therefore, this
45
46 341 parameter is comparable to the detection capabilities calculated in this study.

47
48
49 342 Only one detection limit was claimed by the manufacturer for sulfadiazine (400 µg kg⁻¹). The
50
51 343 detection capability determined here was even lower (300 µg kg⁻¹).

52
53 344 The detection capabilities of Explorer® test for sulphonamides were highThe target concentration
54
55 345 in our study was 100 µg kg⁻¹ for sulphonamides in eggs, like for other edible tissues. Therefore, the
56
57 346 detection capabilities of the 6 tested sulphonamides were between 3 (sulfadiazine,
58
59 347 sulfadimethoxine) and 8 times (sulfamethazine) this target concentration.

1
2 348 As a conclusion, the detection capabilities for sulphonamides of the Explorer® test are
3
4 349 unsatisfactory regarding the target concentration of 100 µg kg⁻¹.

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6 350
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8
9 351 3 different batches of Explorer® test as well as different batches of egg negative samples were
10
11 352 used for the determination of detection capabilities and specificity. There was no observed
12
13 353 influence of the different batches on the results.

14
15 354
16
17 355
18
19
20 356 *Premi®Test.*

21
22
23 357 The detection capabilities calculated for the Premi®Test were lower than those found for the
24
25 358 Explorer® test for sulfadoxine, sulfadimethoxine, sulfamethoxazole, and probably for
26
27 359 sulfamerazine. The detection capabilities of sulfamethazine and sulfadiazine were determined at
28
29 360 200 µg kg⁻¹. Therefore, the detection capabilities of Premi®Test for sulfamethazine and
30
31 361 sulfadiazine seemed to be lower than those of Explorer® test. Finally, Premi®Test was generally
32
33 362 more sensitive for sulphonamides than Explorer® test. One reason could be that egg samples
34
35 363 were diluted before analyses with the Explorer® test as it was recommended by the manufacturer.

36
37
38 364 The detection limits claimed by the manufacturer were 25 µg kg⁻¹ for sulfamethazine, sulfadiazine
39
40 365 and sulfamethoxazole and 100 µg kg⁻¹ for sulfathiazol. The calculated detection capabilities were
41
42 366 higher than those claimed by the manufacturer for SMZ and SDZ. The detection capability for
43
44 367 sulfamethoxazole and sulfathiazole (< 100 and <400 µg kg⁻¹, respectively) could be in accordance
45
46 368 with manufacturer data (25 and 100 µg kg⁻¹, respectively). Like for Explorer® test, the definition of
47
48 369 detection limits was not given by the manufacturer. The calculation of detection limits could be
49
50
51 370 different from the calculation of detection capabilities.

52
53 371 3 different batches of Premi®Test as well as different batches of egg negative samples were used
54
55 372 for the validation. There was no influence of the different batches on the results.

56
57 373
58
59 374 *ELISA kit (from RAISIO Diagnostics)*
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1
2 375 The calculated concentrations for blank samples and all the spiked samples tested with the ELISA
3
4 376 kit were presented in figure 2. The optical densities (OD) (y-axis) are represented in relation to the
5
6 377 number of analyses (x-axis). The 40 analyses per sulphonamide' concentration are duplicate
7
8
9 378 analyses of 20 samples over 4 days. A quick visual reading of the figure showed that the detection
10
11 379 of S6 (sulfamethizole 200 $\mu\text{g kg}^{-1}$.) was unsatisfactory because Optical Denisites were similar to
12
13 380 those of the blank samples.

14
15 381 Insert figure 2

16
17 382 In the ELISA kit leaflet, detection limits (LOD) in eggs were presented. The way to determine the
18
19 383 LOD was to carry out sample blank determinations on at least 20 representative blank samples.
20
21 384 The limit of detection was calculated as: $X_n + 3SD$ ($n \geq 20$) where X_n is the mean response for the
22
23
24 385 20 blank samples. Limits of detection (EC 1993) and detection capabilities (EC 2002) are different
25
26 386 performance characteristics. The decision 2002/657/EC repealed the decision 93/256/EC. These
27
28 387 kits were validated before 2002 and therefore detection limits have been calculated. On the
29
30 388 contrary, detection capabilities are determined by the analysis of at least 20 samples spiked at the
31
32
33 389 lowest detectable concentration, which would give at least 95 % of positive results. Therefore,
34
35 390 limits of detection and detection capabilities could not be compared directly. The parallel that could
36
37 391 be done between LOD and $CC\beta$ is that both of them should be lower than the MRL when MRL
38
39 392 exists and as low as possible when the substance is banned. The $CC\beta$ calculated for the multi-
40
41 393 sulphonamides ELISA test (RAISIO Diagnostics) were equal to or lower than $25 \mu\text{g kg}^{-1}$ for 3 of the
42
43
44 394 6 tested sulphonamides (sulfamethazine, sulfamerazine and sulfisoxazole) and lower than $50 \mu\text{g}$
45
46 395 kg^{-1} for sulfadiazine and sulfachloropyridazine. As a conclusion, the lowest LOD ($3 \mu\text{g kg}^{-1}$)
47
48 396 (sulfamethazine, sulfamerazine and sulfisoxazole) corresponded to the lowest detection
49
50 397 capabilities ($\leq 25 \mu\text{g kg}^{-1}$) and the LOD of $5 \mu\text{g kg}^{-1}$ (sulfadiazine and sulfachloropyridazine)
51
52
53 398 corresponded to the $CC\beta$ of $\leq 50 \mu\text{g kg}^{-1}$. The detection capability of sulfamethizole was higher
54
55 399 than $200 \mu\text{g kg}^{-1}$. The detection capabilities for sulfamethazine and sulfadiazine were lower than
56
57 400 $CC\beta$ of Premi@Test and especially $CC\beta$ of Explorer@ test.

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59
60

1
2 401 For each of the sulphonamide determination of detection capability, 2 different batches were used
3
4 402 during the analyses of the 20 egg samples. There was no observed influence of the batch on the
5
6 403 calculated concentrations (see figure 2). Moreover different boxes of the same batch kit were used.
7

8 404
9
10
11 405 *Other antimicrobials.* The detection capabilities obtained for Premi®Test and Explorer® test for
12
13 406 other antimicrobials are compared in Table IV.

14
15 407 Insert Table IV here
16

17 408
18
19 409 *Explorer® Test*
20

21
22 410 The calculated $CC\beta$ ($> 4000\mu\text{g kg}^{-1}$) was higher than claimed by the manufacturer ($2800\mu\text{g kg}^{-1}$) for
23
24 411 oxytetracycline. However, the calculated detection capabilities were lower than those claimed for
25
26 412 tylosin and tetracycline. The detection capabilities were near the MRL (2 to 2.5 times the MRL) for
27
28 413 macrolides (erythromycin and tylosin). On the contrary, the detection capabilities for tetracyclines
29
30 414 were unsatisfactory regarding the MRL values (7.5 to 20 times the MRL). The detection capability
31
32 415 of doxycycline was very high ($800\mu\text{g kg}^{-1}$) while its use is forbidden for laying hens.

33
34 416 Prior to validation, some analyses were performed at 700 and 1000 $\mu\text{g kg}^{-1}$ with TTC. The
35
36 417 conclusion was that the $CC\beta$ was higher than 1000 $\mu\text{g kg}^{-1}$ (data not shown). Then, 20 repeated
37
38 418 analyses at 1000 $\mu\text{g kg}^{-1}$ and at 1250 $\mu\text{g kg}^{-1}$ with TTC confirmed that $CC\beta$ was higher than 1250
39
40 419 $\mu\text{g kg}^{-1}$ (Table IV). Therefore, $CC\beta$ for tetracycline should be equal to 1500 $\mu\text{g kg}^{-1}$. Oxytetracycline
41
42 420 at 700, 1000 and 2000 $\mu\text{g kg}^{-1}$ were also tested and negative results were obtained at these
43
44 421 concentrations. The detection capability of oxytetracycline was higher than 4000 $\mu\text{g kg}^{-1}$.
45
46 422

47
48 423 There was no MRL for amoxicillin but the detection capability was low ($40\mu\text{g kg}^{-1}$).
49

50
51 424 To our knowledge, no scientific publications exist at this time concerning performance studies of
52
53 425 Explorer® test, neither in eggs nor in other edible tissues. Therefore it was impossible to compare
54
55 426 our results to other scientific data.
56

57 427
58
59
60 428 *Premi®Test*

1
2 428 The calculated detection capabilities were very similar to those claimed by the manufacturer. The
3
4 429 detection capabilities for tetracycline, oxytetracycline, amoxicillin, erythromycin, tylosin and
5
6 430 neomycin were even lower than those claimed. Regarding the regulation, the detection capabilities
7
8
9 431 of Premi®Test were satisfactory for 3 antimicrobial residues (tetracycline, erythromycin, tylosin and
10
11 432 neomycin) because $CC\beta$ were lower than existing MRLs. For oxytetracycline, the $CC\beta$ was equal
12
13 433 to $400 \mu\text{g kg}^{-1}$ and therefore lower than 2 times the MRL. At the MRL, no positive results (out of 20
14
15 434 analyses) were obtained at $200 \mu\text{g kg}^{-1}$. The detection capability of chlortetracycline was high (\leq
16
17 435 $2000 \mu\text{g kg}^{-1}$), when the manufacturer claimed $600 \mu\text{g kg}^{-1}$. Some preliminary assays were
18
19 436 performed to select the antibiotic concentrations to be validated. Chlortetracycline was tested at
20
21
22 437 600 and $800 \mu\text{g kg}^{-1}$. The results were negative. This is the reason why we have decided to
23
24 438 validate at $2000 \mu\text{g kg}^{-1}$. Therefore, the detection capability of chlortetracycline should be included
25
26 439 between 1000 and $2000 \mu\text{g kg}^{-1}$ which was too high regarding the MRL. Microbiological activity
27
28 440 profile was quite different for chlortetracycline from oxytetracycline and tetracycline. The detection
29
30
31 441 capability of amoxicillin was very low ($5 \mu\text{g kg}^{-1}$) and lower than the $CC\beta$ of Explorer® test. This
32
33 442 result was satisfactory because no MRL was set for amoxicillin in eggs. Finally, the detection
34
35 443 capability for doxycycline was 4 times lower with Premi®Test ($200 \mu\text{g kg}^{-1}$) than with Explorer® test
36
37 444 ($800 \mu\text{g kg}^{-1}$). However, considering that this antimicrobial is banned for laying hens, a lower
38
39 445 detection capability would have been preferable.
40
41 446 Like for sulphonamides, the detection capabilities of Premi®Test were much lower than those of
42
43
44 447 Explorer® test. One reason could be that egg samples were diluted before analyses with the
45
46 448 Explorer® test as it was recommended by the manufacturer.
47
48 449 To our knowledge, only 2 studies have been published on the detection of antimicrobial residues in
49
50 450 eggs with Premi®Test (Hussein et al. 2005; Lohajova et al. 2006). Therefore, it was difficult to
51
52 451 compare our results with previous studies. Hussein (2005) reported a comparative study between
53
54 452 a Four Plate Test (FPT) and Premi®Test for the detection of sulfamethazine (SMZ) in laying eggs
55
56
57 453 after treatment of animals. No false positive results were obtained with both tests. Premi®Test was
58
59 454 more sensitive than FPT. The FPT gave positive results when the SMZ concentration was at least
60
455 equal to $31860 \mu\text{g kg}^{-1}$. Negative results were observed at $1720 \mu\text{g kg}^{-1}$. However Premi®Test
456 gave positive results, even at $96 \mu\text{g kg}^{-1}$ of SMZ (8 days after administration). Finally, high

1
2 457 correlation was observed between results of the Premi®Test and HPLC results. In our study, the
3
4 458 detection capability of SMZ in eggs with Premi®Test was higher than $100 \mu\text{g kg}^{-1}$.

5
6 459 A comparison of STAR microbiological method and Premi®Test for the detection of amoxicillin
7
8 460 residues in laying eggs after treatment of animals was also published (Lohajova et al. 2006). Egg
9
10 461 samples were extracted before analyses with the STAR protocol, while no extraction was
11
12 462 performed before Premi®Test. The conclusion was that Premi®Test was a little more sensitive for
13
14 463 amoxicillin than the STAR protocol. In fact, Premi®Test allowed to detect amoxicillin 96h after the
15
16 464 end of the animal treatment in egg yolk and 72h in egg white, while the STAR protocol allowed to
17
18 465 detect amoxicillin after 72h in egg yolk and 48h in egg white after the end of the treatment. Our
19
20 466 validation study also proved that the detection capability of amoxicillin in eggs with Premi®Test
21
22 467 was lower than $5 \mu\text{g kg}^{-1}$ which was also the detection limit claimed by the manufacturer.
23
24
25
26 468

27 28 469 *Cross-reactions.*

29
30
31 470 The percentages (%) of cross-reactivities (CR) were calculated in eggs and were compared with
32
33 471 those claimed by the manufacturer for the ELISA kit (RAISIO Diagnostics) (see Table V). The
34
35 472 calculated % of cross-reactivities were lower than those given by the manufacturer for sulfadiazine,
36
37 473 sulfamerazine and sulfisoxazole. The % CR for sulfachloropyridazine and sulfamethizole are
38
39 474 similar. The % CR of cross-reactivities given by the manufacturer were probably calculated in
40
41 475 buffer, which could explain the differences.
42
43

44 476 Insert Table V here
45
46 477

47 48 478 *Precision.*

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50
51 479 The evaluation of precision was performed with the hypothesis that the MRL for sulphonamides in
52
53 480 eggs would be set at $100 \mu\text{g kg}^{-1}$. The concentrations were lower than those expected because no
54
55 481 recovery was applied to the calculated concentrations given here. The 4 days of analyses were
56
57 482 spread out over 6 months. Moreover, 2 different batches (1 and 2) of RAISIO Diagnostics ELISA
58
59 483 kits were tested (determination of detection capabilities) and different boxes of the same batch kit
60
484 were used. There was no observed influence of the batch on the calculated concentrations. The

1
2 485 overall coefficients of variation were included between 30 and 40 % (Table VI). It is quite high
3
4 486 regarding the criteria of the decision EC/2002/657. However it is acceptable for an ELISA test
5
6 487 which is considered as only a semi-quantitative test.
7

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9 488

Insert Table VI here

10
11 489 These results confirmed that the ELISA kit should have been used to study the stability of
12
13 490 sulphonamides in eggs because of the high variability of quantitative results.
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15 491
16

17 492 *Stability.*
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19
20 493 As it is required in the decision EC/2002/657 (EC 2002), stability studies should be performed
21
22 494 during validation studies. However, the tests implemented in this study were qualitative tests
23
24 495 (Premi®Test, Explorer® test) or only semi-quantitative tests (RAISIO Diagnostics ELISA kits). Our
25
26 496 experience proved that this kind of tests are not suited to perform stability studies. Therefore,
27
28 497 stability data if possible have to be collected in the literature. However, few publications about
29
30
31 498 validation of quantitative methods reported stability data of antimicrobials in food of animal origin,
32
33 499 for example after storage at -20°C . Data on the stability of antimicrobials in eggs were much more
34
35 500 difficult to find than in more common matrices like milk or muscle. None of the validation studies
36
37 501 found in the literature, even for confirmatory methods (physico-chemical), included evaluation of
38
39 502 analyte stability for sulphonamides in eggs. Therefore, stability data of sulphonamides and other
40
41 503 antimicrobials in milk and tissue samples were presented hereafter when no data was found in
42
43
44 504 eggs.

45
46 505 In all studies, the stability of sulphonamide residues in milk at -20°C was proved over 95 days.
47
48 506 Juhel-Gaugain (2005) also demonstrated that there was a problem of stability of sulphonamides in
49
50 507 tissues during storage at -20°C , particularly in liver. This result confirmed the observations from
51
52 508 other studies (Parks et al. 1994). A depletion of the residues at the beginning of the storage at -20
53
54 509 $^{\circ}\text{C}$ (during the first month) was observed. Then, sulfa drugs were stable for a long time (Juhel-
55
56
57 510 Gaugain et al. 2005). Sulfamethazine (SMZ) was found stable in bovine milk and piglet muscle
58
59 511 samples at -20 and -75°C during at least 3 and 5 months, respectively (Papapanagiotou et al.
60
512 2005). However, a trend of decreasing SMZ levels during prolonged storage at -20°C was clear in
513
514 most studies.

1
2 514 The effects of cold storage on the residues of 5 antimicrobials (ampicillin, chloramphenicol,
3
4 515 oxytetracycline, streptomycin and sulfamethazine) in meat at +4°C or -20°C were very variable
5
6 516 (O'Brien et al. 1981). However, the ampicillin concentration in kidney and meat tissues stored at +4
7
8 517 +8C or at -20°C decreased. Stability studies showed that penicillin G and oxytetracycline were
9
10 518 quite stable in incurred tissue samples.

11
12
13 519 At -20°C, oxytetracycline, chloramphenicol and sulfamethazine seemed to be stable. Moreover, in
14
15 520 another study, after 8 months of storage at -20°C, a significant decrease for ampicillin in muscle
16
17 521 arose and was never observed at -75°C (Verdon et al. 2000).

18
19 522 Some articles have been published about the stability of antimicrobials to heat process (cooking).
20
21 523 For example, it was observed that neomycin and streptomycin residues in eggs were quite stable
22
23 524 to normal egg cooking procedures (Katz et al. 1978; Inglis et al. 1978). However, there was no
24
25 525 information about the stability when eggs were stored at +4°C or -20°C for example.
26
27

28 526 The conclusions on this bibliographical study was that there is a huge lack of data on the stability
29
30 527 of antibiotics in eggs. Moreover, when looking at stability studies in other matrices, we could
31
32 528 observe a great variability in the results.
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34 529 35 36 530 *Participation to a proficiency test*

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38
39 531 The results of our lab were satisfactory (Table VII). Firstly because the 2 blank samples were found
40
41 532 negative: hen and quail eggs. Secondly, the samples containing sulphonamide residues have been
42
43 533 found doubtful (material 4a and 4b (SMZ 50 µg kg⁻¹) and material 5 (SDZ 30 µg kg⁻¹)) or even
44
45 534 highly positive (material 3). is the great positivity was a logical result because material 3 contained
46
47 535 a mix of 2 sulphonamides (SDZ 50 µg kg⁻¹ / SDMX 60 µg kg⁻¹). Therefore, the cumulative
48
49 536 concentration was the highest concentration. As a conclusion, the Premi®Test was able to
50
51 537 discriminate between negative blank egg samples and egg samples containing sulphonamide
52
53 538 residues at levels equal or below 50 µg kg⁻¹.
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1
2 540 **Conclusion**

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4
5 541 The majority of approved antibiotics are screened by microbiological methods. Screening tests
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7 542 have to be very fast, easy to perform, with high throughput, enough sensitivity to meet
8
9 543 requirements of regulations (EC 1990) and low-cost. Moreover, wide screening tests have to be
10
11 544 implemented for routine control.

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13 545
14
15 546 The detection capability is the most important characteristic to be determined for a screening test.

16
17 547 The detection capabilities have to be as low as possible for banned substances and lower than

18
19 548 MRL when MRLs have been set. The sensitivity of Premi®Test was better than sensitivity of

20
21 549 Explorer® test for all the tested sulphonamides and the other tested antimicrobials, probably

22
23 550 because of the eggs dilution before Explorer® test. A dilution of egg samples (1/4) before

24
25 551 Explorer® analysis could explain that the detection capabilities are too high for most of the

26
27 552 antimicrobials. The results of our participation to the proficiency test confirmed the performance of

28
29 553 Premi®Test for the detection of sulphonamides in eggs. The detection capabilities for the following

30
31 554 antimicrobials were lower than their respective MRLs: amoxicillin, neomycin, tylosin and

32
33 555 erythromycin. Therefore, Premi®Test seemed to be more suitable to the wide screening of

34
35 556 antimicrobials in eggs as a routine method. Complementary experiments should be implemented to

36
37 557 validate Explorer® test and Premi®Test for more antimicrobial residues.

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39 558
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41
42 559 The second characteristic of a screening test should be to minimise the % of false positive results

43
44 560 to reduce the number of confirmatory analyses. In this case, the false positive rate was very low for

45
46 561 all kits (lower than 2 %) and thus the specificity was satisfactory.

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48 562
49
50
51 563 The multi-sulphonamide ELISA kit is of great interest because generally ELISA tests could detect

52
53 564 only one antibiotic (Spinks et al. 2001). The CC_β values for sulphonamides (25 to 50 µg kg⁻¹) were

54
55 565 much lower than for microbiological tests. This ELISA test should be considered only as a semi-

56
57 566 quantitative test because of high coefficient of variation (precision) (between 30 and 40 %). The

58
59 567 extraction of egg samples was one disadvantage of ELISA kit. It was more time and money

60

1
2 568 consuming than microbiological plate tests and also needed a more skilled technician. So this kind
3
4 569 of ELISA kit could be recommended for the targeted screening of eggs, for sulphonamides
5
6 570 exclusively.
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9 571
10
11 572 Finally, optical reading and microplate format of Explorer® test were advantages versus visual
12
13 573 reading and ampoule format of Premi®Test. High throughput screening could be reach easily.
14
15 574 However, optical reading is also possible for Premi®Test with scanner technology (Stead et al.
16
17 575 2005).
18

19 576
20
21 577 As a conclusion, the control of eggs for the presence of antimicrobials could be reinforced with
22
23 578 these kind of tests because they are simple, rapid and sensitive enough (Premi®Test and ELISA
24
25 579 kit from RAISIO Diagnostics).
26
27

28 580 29 30 581 **Acknowledgements**

31
32
33 582 Thanks to Zeu-Inmunotec (Saragossa, Spain) for providing us with different batches of Explorer®
34
35 583 test, to DSM (Delft, The Netherlands) for providing us with different batches of Premi® test.
36

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2 711 **Figure 1. Example of interpretation of quantitative results (Optical Density) with the**
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4 712 **Explorer® test.**

5
6 713 *This figure represents one day of validation. The optical densities (OD) (difference between the*
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8
9 714 *values of the 2 readings at 2 wavelengths (590 and 650 nm)) are represented on y-axis in relation*
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11 715 *to the number of analyses on x-axis. The difference of OD of blank samples (negative control NA)*
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13 716 *are represented with a square shape ■. The difference of OD of positive control (penicillin G at 25*
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15 717 *µg kg⁻¹) are represented with a triangle shape ▲. The negative control sample absorbance (NA) +*
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17 718 *0.15 is represented with a cross shape ×. The result (SA = Sample Absorbance) of each unknown*
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19
20 719 *sample (diamond shape ◆) was compared to this line. A sample was declared positive when: SA*
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22 720 *590nm - SA 650 nm ≥ NA590nm - NA 650nm + 0.15.*

23
24 721
25
26 722 **Figure 2. Determination of detection capabilities for the multi-sulphonamides ELISA kit**
27
28 723 **(RAISIO Diagnostics).**

29
30
31 724 *This figure represents the optical densities (OD) (y-axis) in relation to the number of analyses (x-*
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33 725 *axis). The OD of blank samples are represented with a diamond shape ◆ and the points are linked*
34
35 726 *by a line.*

36
37 727 *The 40 analyses per sulphonamide' concentration are duplicate analyses of 20 samples over 4*
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39 728 *days. The 6 sulphonamides are as follows: S1 = sulfamethazine 25 µg kg⁻¹, S2 = sulfadiazine 50*
40
41 729 *µg kg⁻¹, S3 = sulfamerazine 25 µg kg⁻¹, S4 = sulfisoxazole 25 µg kg⁻¹, S5 = sulfachloropyridazine*
42
43
44 730 *50 µg kg⁻¹ and S6 = sulfamethizole 200 µg kg⁻¹.*

Figure 1.

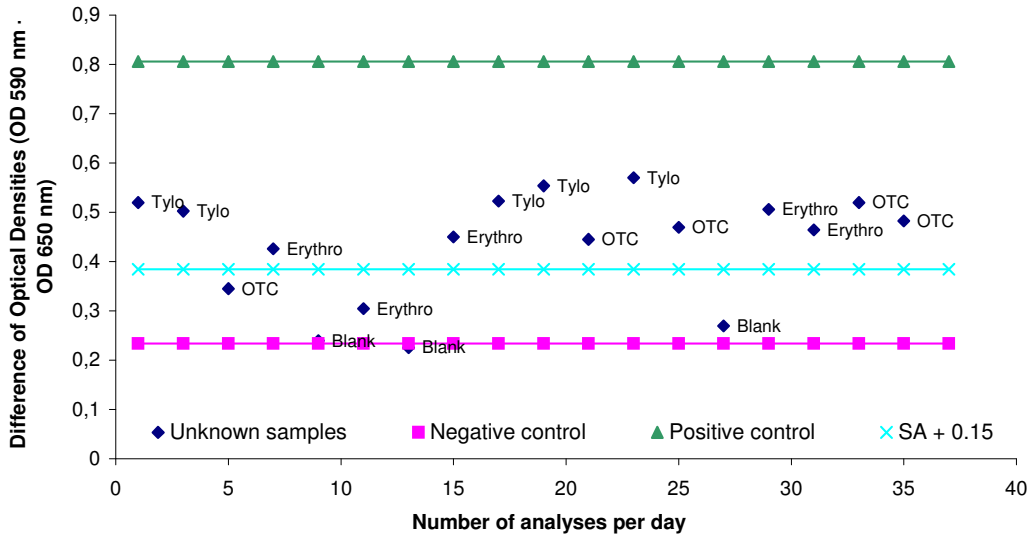
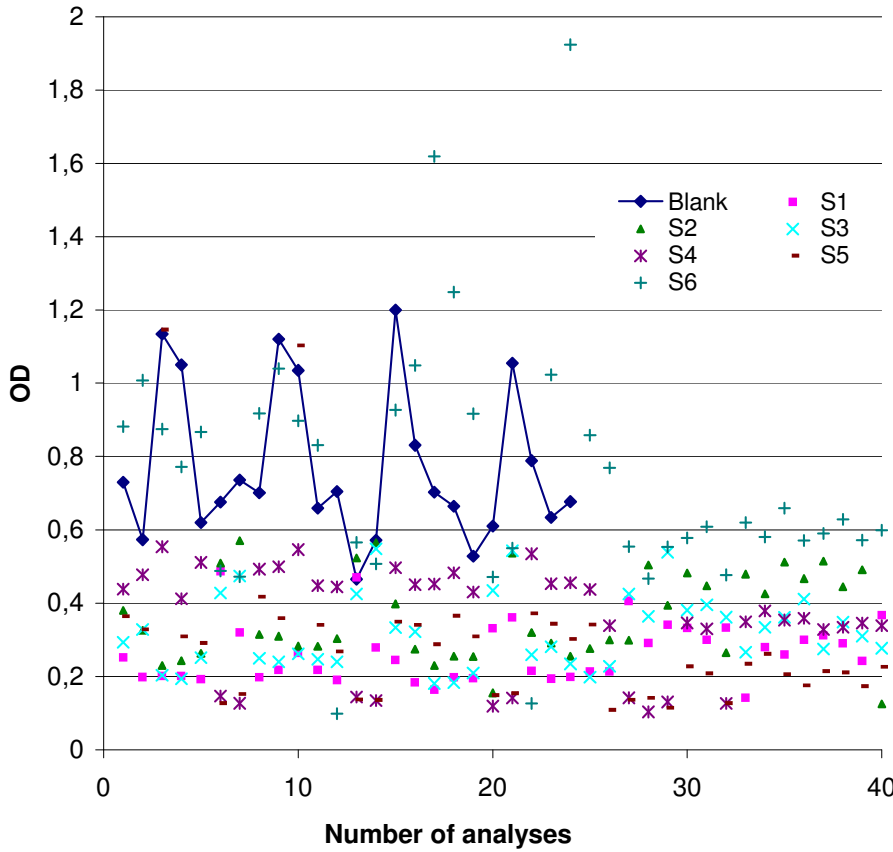


Figure 2.



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1 Table I. Antimicrobial substances with their respective MRLs in eggs.

AB	Marker residue	Species	MRL ($\mu\text{g kg}^{-1}$)
Chlortetracycline	Sum of parent drug and its 4-epimer	All food producing species	200
Oxytetracycline			
Tetracycline			
Erythromycin	Erythromycin A	All food producing species	150
Tylosin	Tylosin A	All food producing species	200
Neomycin (including framycetin)	Neomycin B	All food producing species	500
Lincomycin	Lincomycin	All food producing species	50
Tiamulin	Tiamulin	Chicken	1000
Colistin	Colistin	All food producing species	300

4 Table II. Antimicrobial substances tested with the 3 kits.

		Tested concentrations (ng g ⁻¹)		
SULPHONAMIDES	MRL	Premi®Test	Explorer®	ELISA (RAISIO Diagnostics)
Sulfamethazine	/*	100	800	25
Sulfadiazine	/	100	200	50
Sulfamerazine	/	100	500	25
Sulfisoxazole	/	/	/	25
Sulfachloropyridazine	/	100	/	50
Sulfamethizole	/	100	/	200
Sulfathiazol	/	400	/	/
Sulfadoxine	/	100	500	/
Sulfadimethoxine	/	100	500	/
Sulfamethoxazole	/	100	500	/

		Tested concentrations (ng g ⁻¹)		
OTHER ANTIMICROBIALS	MRL	Premi®Test	Explorer®	ELISA (RAISIO Diagnostics)
Chlortetracycline (CTC)	200	2000	/	/
Tetracycline (TTC)	200	200	800	/
Oxytetracycline (OTC)	200	400	3000- 4000	/
Doxycycline	/	200	1500	/
Amoxicillin	/	5	40	/
Erythromycin	200	50	300-400	/
Tylosin	100	50	200-250	/
Neomycin	500	300	/	/

5 *No MRL has been set for sulphonamides in eggs at this time.*

7 **Table III. Comparison of the detection capabilities calculated for the 3 kits for sulphonamides and**
 8 **comparison with the announced detection limits.**

	Premi®Test			Explorer®			RAISIO Diagnostics®	
	Levels of fortification ($\mu\text{g kg}^{-1}$)	nb + results /spiked samples	CC β	Levels of fortification ($\mu\text{g kg}^{-1}$)	nb + results /spiked samples	CC β	Levels of fortification ($\mu\text{g kg}^{-1}$)	CC β
Sulfamethazine	100 200	25/40 20/20	>100 200	800	19/20	800	25	≤ 25
Sulfadiazine	100 200	35/40 20/20	>100 200	200 300	16/20 19/20	>200 300	50	≤ 50
Sulfamerazine	/	NT	NT	250 350 500	15/20 20/20 20/20	>250 350 ≤ 500	25	≤ 25
Sulfadoxine	100	20/20	≤ 100	500	16/20	> 500	/	NT
Sulfadimethoxine	100	20/20	≤ 100	250 300 500	18/20 20/20 20/20	>250 300 ≤ 500	/	NT
Sulfamethoxazole	100	25/25	≤ 100	500	19/20	500	/	NT
Sulfachloropyridazine	/	NT	NT	/	NT	NT	50	≤ 50
Sulfamethizole	/	NT	NT	/	NT	NT	200	> 200
Sulfathiazol	400	20/20	≤ 400	/	NT	NT	/	NT
Sulfisoxazole	/	NT	NT	/	NT	NT	25	≤ 25

* 20/20 (number of positive results / number of spiked samples analysed) for all tested sulphonamides.

1
2 13 **Table IV. Comparison of the detection capabilities calculated for Premi®Test and Explorer® test for**
3
4 14 **the other antimicrobial residues and comparison with the announced detection limits.**
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6 15

	Premi®Test					Explorer®			
	MRL	Announced LOD (DSM) ($\mu\text{g kg}^{-1}$)	Levels of fortification ($\mu\text{g kg}^{-1}$)	nb + results /spiked samples	CC β ($\mu\text{g kg}^{-1}$)	Announced LOD (Zeu) ($\mu\text{g kg}^{-1}$)	Levels of fortification ($\mu\text{g kg}^{-1}$)	nb + results /spiked samples	CC β ($\mu\text{g kg}^{-1}$)
CTC	200	600	2000	20/20	≤ 2000	/	/	/	/
TTC	200	200	200	20/20	≤ 200	2800	1000 1250 1500	8/20 16/20 20/20	>1000 >1250 1500
OTC	200	400	200 400	0/20 20/20	400	2800	3000 4000	14/20 15/20	>4000
Doxycycline	/	200	100 200	0/20 20/20	100 200	/	800	19/20	800
Amoxicillin	/	5	5	19/20	5	/	40	20/20	≤ 40
Erythromycin	200	50	50	20/20	≤ 50	400	200 300 400	7/20 14/20 20/20	>200 >300 400
Tylosin	100	50	50	20/20	≤ 50	400	200 250	15/20 20/20	>200 250
Neomycin	500	300	300	19/20	300	1600	/	/	/

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17 *LOD: detection limit*
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2 19 **Table V. Cross-reactivity percentages.**

	Announced % CR	Calculated % CR
S1 : Sulfamethazine (SMZ)	100*	100*
S2 : Sulfadiazine	68	36.6
S3 : Sulfamerazine	108	77.9
S4 : Sulfisoxazole	99	68.6
S5 : Sulfachloropyridazine	64	52.3
S6 : Sulfamethizole	5.3	3.6
Sulfathiazol	7	NT
Sulfadoxine	<1	NT
Sulfadimethoxine	<1	NT
Sulfachloropyrazine	97	NT

18 20 *S1 to S6 were tested with the multi-sulphonamides ELISA kit.*

20 21 ** SMZ is the reference sulphonamide to calculate cross-reactivity percentages.*

22 Table VI. Precision determined for the multi-sulphonamides ELISA kit (RAISIO Diagnostics).

		D3+D5	D2	D4+D6	D7	
Batch		1	2	1	2	
Box		4	9	5	9-10	
						TOTAL
0.5 * target concentratio n	Mean	20	27	43	22	28
	SD	8	1	5	1	10
	CV	40	4	12	5	37
target concentratio n	Mean	41	51	69	32	50
	SD	9	10	6	3	16
	CV	23	20	8	8	31
1.5 target concentratio n	Mean	51	51	80	NC*	61
	SD	19	11	8	NC	19
	CV	38	21	10	NC	31

24 Target concentration was $100 \mu\text{g kg}^{-1}$.

26 D2: Day 2 of validation

28 Mean: mean concentration (intra-day $n=6$; inter-days $n=36$)

30 SD: standard deviation (intra-day $n=6$; inter-days $n=36$)

32 CV: Coefficient of variation (%)

34 * NC: not calculated because no quantitative results (results were out of the standard curve)

36 Batch: 2 different batches supplied by the manufacturer have been tested (different batch number, different validity date)

38 Box: From each batch, different boxes (each containing a microplate 96 wells and reagents) were also tested (different boxes with the same batch number and the same validity date).

33 **Table VII. Results of our participation to the proficiency test for the detection of sulphonamides in**
 34 **eggs with Premi®Test.**

	Material 1	Material 2	Material3	Material 4a	Material 4b	Material 5
AB	Blank laying hen	Blank quail egg	SDZ / SDMX	SMZ	SMZ	SDZ
Concentration ($\mu\text{g kg}^{-1}$)	/	/	50 / 60	50	50	30
PT results	-	-	++	D	D	D

35
 36 *sulfadiazine (SDZ), sulfadimethoxine (SDMX), sulfamethazine (SMZ)*

37 *D: Doubtful*