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Comparative study of three screening tests, two microbiological tube tests and a multi-sulphonamide ELISA kit, for the detection of antimicrobial and sulphonamide residues in eggs

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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.*

1
2 39 **Introduction**

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4 40 Eggs are a staple food in the world for many populations providing a vital sources of animal
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6 41 protein. Antimicrobials are used as feed additives or in drinking water in poultry breeding to prevent
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8 42 animal diseases (enteric diseases) (Dahiya et al. 2006) or as growth promoting factors (Donoghue
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10 43 2003; Stolker et al. 2007). The use of antimicrobials in laying hens could result in the persistence
11
12 44 of residues in eggs. Different studies of the excretion of tetracyclines and their epimers have been
13
14 45 performed after oral medication of laying hens (Roudaut et al. 1989; Zurhelle et al. 2000; Donkova
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16 46 et al. 2005), macrolides (Roudaut et al. 1990); sulphonamides (Shaikh et al. 2000; Roudaut 2002;
17
18 47 Shaikh et al. 2004), ampicillin (Donoghue et al. 1997). The conclusion was that residues of
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20 48 antimicrobials could persist in eggs several days after medication in drinking water. Furthermore,
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22 49 Kan (2000) reviewed several studies on drug residues in eggs and their distribution between yolk
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24 50 and white, after administration to laying hens. Some models were established to evaluate the
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26 51 persistence of antimicrobial residues in eggs. A sulfamethazine model was presented by Tansakul
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28 52 (2007) which demonstrated that even low doses of sulfamethazine could result in the presence of
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30 53 residues in eggs and therefore could present a risk for consumer safety. As a conclusion, nearly all
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32 54 veterinary drugs and feed additives available on the market may result in residues in eggs (Kan et
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34 55 al. 2007).

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41 57 Therefore, the detection of antimicrobial residues in eggs is especially of great interest both for its
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43 58 impact on public health. Moreover, the presence of residues could lead to bacterial resistance to
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45 59 different antimicrobials (i.e. streptomycin and tetracycline) (Adesiyun et al. 2007). A study
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47 60 conducted by the Canadian Food Inspection Agency (CFIA) reported the level of contamination of
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49 61 eggs with antimicrobials (Quon 2000). This study was conducted over a period of 2 years on both
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51 62 domestic and imported eggs. More than 99% of the samples screened were found to be free of any
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53 63 veterinary drug residue. The other samples contained tetracyclines, sulphonamides, ciprofloxacin,
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55 64 macrolides, and streptomycin. Another study conducted in Trinidad concluded that the
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57 65 antimicrobial residues detected in table eggs were particularly sulphonamides and then
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59 66 macrolides, tetracyclines, and beta-lactam (Adesiyun et al. 2005). Sulphonamides are still effective
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61 67 tools in the treatment of coccidiosis in broiler chicken and are widely used in intensive poultry

farms. The Committee for Veterinary Medicinal Products recommended that the sulphonamides are entered into Annex I of Council Regulation (EEC) 2377/90 (EC 1990). The MRL of sulphonamides for meat and milk is 100 $\mu\text{g kg}^{-1}$. However, there is no MRL set for sulphonamide in egg products. In fact, there are no sulphonamides and no beta-lactams approved for use in laying hens. Among macrolides, tylosin is intended to be administered to laying hens, in the drinking water or in premix formulations, for the treatment of respiratory diseases and necrotic enteritis. Erythromycin is used for the treatment of chronic respiratory diseases due to mycoplasma in poultry. In veterinary medicine, tetracyclines are allowed for medication of laying hens and used as a broad spectrum antibiotic. Neomycin is used to treat bacterial gastrointestinal infections of poultry by the oral route. The previously cited antimicrobials are authorised to be administered to laying hens. The European Union has identified Maximum Residue Limits (MRL) for some molecules but very few antimicrobials have MRL in eggs (Table I).

Insert Table I here.

When the use of some antimicrobials is forbidden for all animal species (i.e. chloramphenicol), they are entered in Annex IV of the Regulation EC 2377/90 (EC 1990). Furthermore, some antimicrobials are forbidden to be administered to laying hens produced for human consumption only (i.e. doxycycline, spectinomycin, enrofloxacin). However, other antimicrobials (i.e. penicillins, sulphonamides) have no MRL set for eggs (thus a “zero” tolerance). Therefore, in both cases, the detection capabilities of the methods have to be as low as possible as a consequence of no authorisation of these antimicrobials in eggs and no MRL set for this product.

Due to the possible persistence of antimicrobial residues in eggs, the presence of antibiotics must be controlled with relevant analytical methods to meet the EU regulations. There are very few publications at this time describing screening methods for the detection of antimicrobial in eggs. However, the screening step is the first step of the control and thus is an essential step. There is a need of wide spectrum screening tests, quick, easy to use and cheap. Different screening tests were developed for the screening of antimicrobials in eggs: microbiological plate tests (Kabir et al. 2004; Bendix et al. 2005; Pikkemaat et al. 2007), tube tests like Premi®Test (Hussein et al. 2005) 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
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4 98 et al. 2005)) or physicochemical methods like HPLC (Horii et al. 1990; Herranz et al. 2007) and
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6 99 LC/MS-MS methods (Wang et al. 2005; Heller et al. 2006; Stolker et al. 2007). To our knowledge,
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8 100 there is no scientific publication at this time describing the use of Explorer® test for the screening
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11 101 of antimicrobials in eggs.
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15 103 An interlaboratory study was organised for the screening and the confirmation of different
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17 104 sulphonamides in eggs by our laboratory as Community Reference Laboratory. On this occasion, a
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19 105 study was conducted to evaluate the performances of 3 different commercial kits for their ability to
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21 106 detect sulphonamides and other antimicrobials in eggs: one ELISA kit multi-sulphonamides (from
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23 107 RAISIO Diagnostics, Diffchamb, Lyon, France) and 2 microbiological tests (Premi®Test from DSM,
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25 108 Delft, The Netherlands and Explorer® kit from Zeu-Inmunotec, Saragossa, Spain). Premi®Test
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27 109 and Explorer® test are 2 commercial microbiological based tests. These kits, based on the
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29 110 inhibition of bacterial growth (*Bacillus stearothermophilus* strain), contain a redox indicator that
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31 111 changes colour in the absence of antibiotics in the sample. They are simple and fast methods for
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33 112 the detection of antibiotics in fresh meat, eggs and feed samples. Technical guidelines and
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35 113 performance criteria (e.g., detection capability CC_β, selectivity and specificity) for residue control in
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37 114 the framework of Directive 96/23/EC (EC 1996) are described in Commission Decision
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39 115 2002/657/EC (EC 2002). This paper reports the validation of 3 kits for the detection of
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41 116 sulphonamides and other antimicrobials in egg samples according to the Commission Decision
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43 117 2002/657/EC (EC 2002).
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50 119 **Materials and methods**

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53 120 *Explorer® test*
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56 121 Explorer® test is a commercial kit for detection of inhibitory substances in raw meat and eggs. The
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58 122 test, a 96-well microtiter plate, is based on the inhibition of microbial growth. Each well contains
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60 123 specific agar medium containing a redox indicator and spread with *Geobacillus stearothermophilus*
124 spores.

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

Test procedure. 100 µl of diluted egg was added to each well. The wells were sealed with a plastic film and the microplate was pre-incubated at 65°C for 30 min in a block heater (Zeu-Inmunotec, Spain) and the sample allowed to diffuse through the well (pre-diffusion). Afterwards the wells were washed by filling the wells up with distilled water, using a squeeze bottle. The washing step was repeated 3 times to 4 times. The plate was emptied by turning the plate upside down on top of an absorbent paper to remove the water in excess. NB: It should not remain traces of coagulated egg!! The plate was sealed again with plastic wrap and incubated at 65°C until the negative control sample has turned orange (approximately 3h-3h30min).

Readings and interpretation of Explorer® results.

The photometric reading of microplates is based on the reading at 2 wavelengths (590 and 650 nm) on a microplate reader. The results were interpreted as the difference between the values of the two readings. On each day of validation, one negative control was analysed and also one positive control (penicillin G at 25 µg kg⁻¹) (Quality Control QC). The plate is ready to be read when the result for the negative control sample (difference of absorbance NA590nm - NA650 nm) is between 0.15 and 0.25 OD (optical density units), where NA: Negative control absorbance. A sample is declared positive when: SA 590nm - SA 650 nm ≥ NA590nm - NA 650nm + 0.15, where : SA: Sample absorbance.

One single day of validation is presented as an example of interpretation. The result of each unknown sample (SA = Sample Absorbance) is compared to the line of negative control sample absorbance (NA) + 0.15. When: SA 590nm - SA 650 nm ≥ NA590nm - NA 650nm + 0.15, the

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

Insert figure 1

Premi® test

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

Preparation of egg samples. Egg sample was homogenised (white and yolk).

Test procedure. A syringe with a set volume of 100 µl is supplied with the kit. 100 µl of homogenized egg fluid was transferred onto the agar in the ampoule, by pressing the syringe and releasing it. The ampoule is closed with the plastic foil supplied with the kit. The ampoule(s) was placed in a water bath at 80°C for 10 minutes. After this heat pre-treatment, the ampoules were incubated at the required temperature of 64°C ($\pm 0,5$ °C). The ampoules were incubated until the negative control (egg fluid what has been tested before negative) changed colour (turned yellow).

Readings and interpretation of Premi®Test results. When the negative control changes colour from purple to yellow (approx. 3 hours), the results could be read from the bottom 2/3 part of the ampoule. A clear colour change purple to yellow indicates that the antimicrobial compounds were below the Premi®Test detection limits. A purple colour indicates the presence of antibiotics at or above the detection limits of the Premi®Test.

Multi-sulphonamides ELISA kit (RAISIO Diagnostics)

This commercial multi-sulphonamide test is a competitive enzyme immunoassay (EIA) for quantitative screening of tissue, milk, honey, egg and urine samples. The method should detect a wide range of sulphonamides. It is based on monoclonal antibodies (21C7) raised against protein bound sulfamethazine. The manufacturer claimed that it offers high sensitivity for sulfamethazine, sulfadiazine, sulfamerazine, sulfisoxazole, sulfachloropyridazine and sulfachloropyrazine. This kit is suitable for screening tissue, egg, milk, honey and urine for sulphonamides.

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

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

Test procedure. 100 µl of the zero standard in duplicate (wells A1, A2, blank), 50 µl of the zero standard in duplicate (wells B1, B2, maximal signal), 50 µl of each of the sulfamethazine standard 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 each extracted sample in duplicate were pipetted. Then 25 µl conjugate (sulphonamide-HRPO) and 25 µl antibody solution were added to all wells, except wells A1 and A2. The microtiter plate was sealed and shaken for a few seconds. The plate was incubated for 1 hour in the dark between +2°C and +8°C). The microtiter plate was washed 3 times with rinsing buffer. Then 100 µl substrate/chromogen solution was added to all wells. The plate was again incubated, 30 min at room temperature in the dark. Finally the reaction was sopped by adding 100 µl of stop solution to each well.

Readings and interpretation of ELISA Test results. Immediately after adding the stop solution, the absorbance values at 450 nm was read. The values (% maximal absorbance) calculated for the standards are plotted (on the Y-axis) versus the sulphonamide equivalent concentration (ng ml⁻¹) on a logarithmic x-axis. The amount of sulphonamide in the samples is expressed as sulphonamide equivalents. The sulphonamide equivalents in the extracts (ng ml⁻¹) corresponding to the % maximal absorbance of each extract can be read from the calibration curve.

Quality controls (QC)

As it was strongly recommended by the manufacturer ((Premi®Test, DSM and Explorer®, Zeu-
Inmunotec), a negative control sample (antibiotic-free egg) was used to determine the optimal
incubation time of each assay. Moreover, a positive sample (egg sample spiked with high
concentration of antibiotic, ie. Penicillin G 25 µg kg⁻¹) was tested at each day of validation to check
the performance of the kit.

Validation

Specificity and false positive rate. To determine these parameters, at least 20 egg samples from
different origins were analysed in blind duplicate for each of the 3 kits. Finally, even more than 20
blank egg samples were analysed with Premi®Test (33 blank egg samples) and with Explorer®
test (55 blank egg samples) (1 to 3 were analysed at each day of validation). However, only 12
blank samples have been analysed at the end with the ELISA kit. In practice, some experiments
failed because of stability issues of the conjugate. The instability of the conjugate was observed
when some days the optical density of the blank standard was lower than 0.6 OD, which was the
validity limit for the kit. For this reason, the number of negative samples that could be analysed
should be reduced to 12 samples only.

Detection capabilities. The concentration level, where only ≤ 5 % false compliant results remain,
equals the detection capability of the method (EC 2002).

A preliminary study allowed us to choose for all the 3 kits, the target concentrations for each
sulphonamide and each antimicrobial. It is the least detectable concentration in eggs. Then, at
least 20 egg samples were spiked for each sulphonamide and each antimicrobial, for at least one
concentration level (MRL or claimed detection capability). 20 analyses have to be carried out in
order to ensure a reliable basis for this determination. These samples were analysed in blind
duplicate with each test.

The RAISIO Diagnostics ELISA kit was a multi-sulphonamides ELISA kit which was not able to
detect other antimicrobial residues. Therefore only sulphonamides have been tested for their
detection by the ELISA kit.

Selection of the antimicrobial compounds and concentrations. The sulphonamides (sulfamethazine (SMZ), sulfadiazine (SDZ), sulfamethoxazole, sulfadoxine, sulfamerazine, sulfadimethoxine, sulfisoxazole, sulfachloropyridazine, sulfamethizole, sulfathiazol) and the tested concentrations were selected by the CRL according to the data given by the 3 manufacturers on detection limits. The other antimicrobials were chosen because some of them have MRL in eggs (chlortetracycline (CTC), oxytetracycline (OTC), tetracycline (TTC), erythromycin, tylosin and neomycin). The others (doxycycline, amoxicillin) were selected because they are from the main families of antimicrobials (Table II).

Insert Table II here

For preparation of antibiotic stock solutions, antibiotic standards with known purity were used (from Sigma Aldrich, France; Cluzeau InfoLabo, France). The stock solutions were diluted with distilled water to prepare spiked samples.

Cross-reactivities. The percentages of cross-reactivities between SMZ and other sulphonamides were only calculated for the multi-sulphonamides ELISA kit when determining the detection capability CC_β. The cross-reactivity percentages (%) will be compared with those claimed by the manufacturer.

Precision. The precision was determined as it is written in the European Decision EC/2002/657 (EC 2002) for the RAISIO Diagnostics ELISA kit which was the only quantitative test. In this decision, the precision has to be determined for quantitative screening tests. For authorised substances (MRL), the precision has to be determined at 0.5, 1 and 1.5 times the MRL. For banned substances, the precision has to be determined at 1, 1.5 and 2 times the MRPL, where MRPL is the Minimum Required Performance Limit. Concerning the detection of sulphonamides in eggs, we have no MRL and no MRPL. In the literature, validation studies for sulphonamides in eggs are commonly based on a target concentration of 100 µg kg⁻¹ (Tarbin et al. 1999; Heller et al. 2006; Wang et al. 2007). Therefore, the evaluation of precision was performed with a target concentration of 100 µg kg⁻¹, as it was the MRL value at. Two samples of blank egg samples were

spiked with sulfamethazine solutions to yield concentrations equivalent to 0.5, 1 and 1.5 times the MRL. At each level, the analysis at least six replicate analyses were performed each day of analysis. These steps were repeated on 4 days. The SMZ concentration detected in each sample was calculated. Finally, the mean concentration, standard deviation (SD) and the coefficient of variation (CV %) of the fortified samples were calculated. Different experiment were performed: Same batch, same technician (repeatability conditions) (2 days) and other batch, same technician (reproducibility conditions) (2 days).

Preparation of spiked egg samples. Egg samples were spiked on each day of validation. 20 µl of each antimicrobial solution was added to 2 g of egg sample. All the samples were codified to perform blind analyses.

Stability. No experiments have been carried out to determine the stability of sulphonamides or other antimicrobials in eggs. A stability study of different antibiotics in solution and in kidney juices was already published (Cantwell et al. 2006). However, because the 3 tests could not distinguish between all sulphonamides, the stability study should have been performed for all detected sulphonamides in separate experiments. Furthermore, Premi®Test and Explorer® test could not distinguish between all detected antibiotics. The number of experiments would have been high. This kind of study would have become expensive and the workload would have been very high. Finally, the ELISA kit (from RAISIO Diagnostics) allowed to report quantitative results. However, our experience showed that ELISA kits and other immunological methods like BIACORE methods (Gaudin et al. 2007) are not fitted to test stability of drugs because of their high variability. Therefore, the stability of the analytes in the egg matrix was studied by a bibliographical study.

Participation to a proficiency test

A proficiency test (PT) was organised by the Community Reference Laboratory (CRL) in Fougères to evaluate the participants' methods to detect, confirm and quantify residues of sulphonamides in whole eggs. The participants were free to use the method of their choice. Therefore several

different methods have been implemented (in house methods or commercial kits). The participants were National Reference Laboratories (NRLs) from Member States and Third Countries. As the French NRL, our laboratory has participated to the PT with the Premi®Test. However, the technicians who implemented the Premi®Test were not involved in the organisation of the PT and did not know the content of samples. Six samples were sent to the participants in December 2006, 2 blank samples and 3 spiked samples (materials 3, 4, 5). 2 blank samples were sent (1 laying hens (material 1) and 1 quail egg (material 2)). Material 3 contained sulfadiazine (SDZ) at 50 $\mu\text{g kg}^{-1}$ and sulfadimethoxine (SDMX) at 60 $\mu\text{g kg}^{-1}$, material 4 contained sulfamethazine (SMZ) at 50 $\mu\text{g kg}^{-1}$ and material 5 contained sulfadiazine at 30 $\mu\text{g kg}^{-1}$. Material 4 (a/b) was sent in blind duplicate.

Results and discussion

Specificity and false positive rate

Explorer® test. Only one false positive result was obtained out of 55 blank egg samples. Therefore, the false positive rate was satisfactory (1.82%). 2 different batches of Explorer® were used during this study. Moreover, 2 different batches of eggs were also analysed.

Premi®Test. 33 blank egg samples have been tested finally. The false positive rate was 0%.

ELISA kit (RAISIO Diagnostics). For the 12 blank egg samples, calculated SMZ concentrations were very low (between 0.0 +/- 0.0 and 4.5 +/- 1.9 $\mu\text{g kg}^{-1}$ over the 6 days). The overall mean was 2.2 +/- 2.0 $\mu\text{g kg}^{-1}$. The distribution of the SMZ concentrations of the 12 blank samples was represented on figure 1.

Insert Figure 1

The difference of calculated SMZ concentrations between blank samples and spiked SMZ samples at 25 $\mu\text{g kg}^{-1}$ is clearly observable. No false positive results were observed.

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

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13 326 *Detection capabilities.*

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

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26 332 *Sulphonamides.* The CC_β values obtained for the 3 kits for sulphonamides are compared in Table
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29 333 III.

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31 334 Insert Table III here

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33 335 *Explorer® Test.*

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

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

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

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

3 different batches of Explorer® test as well as different batches of egg negative samples were used for the determination of detection capabilities and specificity. There was no observed influence of the different batches on the results.

Premi®Test.

The detection capabilities calculated for the Premi®Test were lower than those found for the Explorer® test for sulfadoxine, sulfadimethoxine, sulfamethoxazole, and probably for sulfamerazine. The detection capabilities of sulfamethazine and sulfadiazine were determined at 200 µg kg⁻¹. Therefore, the detection capabilities of Premi®Test for sulfamethazine and sulfadiazine seemed to be lower than those of Explorer® test. Finally, Premi®Test was generally more sensitive for sulphonamides than Explorer® test. One reason could be that egg samples were diluted before analyses with the Explorer® test as it was recommended by the manufacturer. The detection limits claimed by the manufacturer were 25 µg kg⁻¹ for sulfamethazine, sulfadiazine and sulfamethoxazole and 100 µg kg⁻¹ for sulfathiazol. The calculated detection capabilities were higher than those claimed by the manufacturer for SMZ and SDZ. The detection capability for sulfamethoxazole and sulfathiazole (< 100 and <400 µg kg⁻¹, respectively) could be in accordance with manufacturer data (25 and 100 µg kg⁻¹, respectively). Like for Explorer® test, the definition of detection limits was not given by the manufacturer. The calculation of detection limits could be different from the calculation of detection capabilities.

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

ELISA kit (from RAISIO Diagnostics)

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2 375 The calculated concentrations for blank samples and all the spiked samples tested with the ELISA
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4 376 kit were presented in figure 2. The optical densities (OD) (y-axis) are represented in relation to the
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6 377 number of analyses (x-axis). The 40 analyses per sulphonamide' concentration are duplicate
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9 378 analyses of 20 samples over 4 days. A quick visual reading of the figure showed that the detection
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11 379 of S6 (sulfamethizole 200 µg kg⁻¹.) was unsatisfactory because Optical Denisites were similar to
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13 380 those of the blank samples.

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15 381 Insert figure 2

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17 382 In the ELISA kit leaflet, detection limits (LOD) in eggs were presented. The way to determine the
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19 383 LOD was to carry out sample blank determinations on at least 20 representative blank samples.
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21 384 The limit of detection was calculated as: $X_n + 3SD$ ($n \geq 20$) where X_n is the mean response for the
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23
24 385 20 blank samples. Limits of detection (EC 1993) and detection capabilities (EC 2002) are different
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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
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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-
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41 393 sulphonamides ELISA test (RAISIO Diagnostics) were equal to or lower than 25 µg kg⁻¹ for 3 of the
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43
44 394 6 tested sulphonamides (sulfamethazine, sulfamerazine and sulfisoxazole) and lower than 50 µg
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46 395 kg⁻¹ for sulfadiazine and sulfachloropyridazine. As a conclusion, the lowest LOD (3 µg kg⁻¹)
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48 396 (sulfamethazine, sulfamerazine and sulfisoxazole) corresponded to the lowest detection
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50 397 capabilities (≤ 25 µg kg⁻¹) and the LOD of 5 µg kg⁻¹ (sulfadiazine and sulfachloropyridazine)
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53 398 corresponded to the $CC\beta$ of ≤ 50 µg kg⁻¹. The detection capability of sulfamethizole was higher
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55 399 than 200 µg kg⁻¹. The detection capabilities for sulfamethazine and sulfadiazine were lower than
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57 400 $CC\beta$ of Premi®Test and especially $CC\beta$ of Explorer® test.

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

Other antimicrobials. The detection capabilities obtained for Premi®Test and Explorer® test for other antimicrobials are compared in Table IV.

Insert Table IV here

Explorer® Test

The calculated CC β ($> 4000 \mu\text{g kg}^{-1}$) was higher than claimed by the manufacturer ($2800 \mu\text{g kg}^{-1}$) for oxytetracycline. However, the calculated detection capabilities were lower than those claimed for tylosin and tetracycline. The detection capabilities were near the MRL (2 to 2.5 times the MRL) for macrolides (erythromycin and tylosin). On the contrary, the detection capabilities for tetracyclines were unsatisfactory regarding the MRL values (7.5 to 20 times the MRL). The detection capability of doxycycline was very high ($800 \mu\text{g kg}^{-1}$) while its use is forbidden for laying hens.

Prior to validation, some analyses were performed at 700 and $1000 \mu\text{g kg}^{-1}$ with TTC. The conclusion was that the CC β was higher than $1000 \mu\text{g kg}^{-1}$ (data not shown). Then, 20 repeated analyses at $1000 \mu\text{g kg}^{-1}$ and at $1250 \mu\text{g kg}^{-1}$ with TTC confirmed that CC β was higher than $1250 \mu\text{g kg}^{-1}$ (Table IV). Therefore, CC β for tetracycline should be equal to $1500 \mu\text{g kg}^{-1}$. Oxytetracycline at 700, 1000 and $2000 \mu\text{g kg}^{-1}$ were also tested and negative results were obtained at these concentrations. The detection capability of oxytetracycline was higher than $4000 \mu\text{g kg}^{-1}$.

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

To our knowledge, no scientific publications exist at this time concerning performance studies of Explorer® test, neither in eggs nor in other edible tissues. Therefore it was impossible to compare our results to other scientific data.

Premi®Test

The calculated detection capabilities were very similar to those claimed by the manufacturer. The detection capabilities for tetracycline, oxytetracycline, amoxicillin, erythromycin, tylosin and neomycin were even lower than those claimed. Regarding the regulation, the detection capabilities of Premi®Test were satisfactory for 3 antimicrobial residues (tetracycline, erythromycin, tylosin and neomycin) because CC β were lower than existing MRLs. For oxytetracycline, the CC β was equal to 400 $\mu\text{g kg}^{-1}$ and therefore lower than 2 times the MRL. At the MRL, no positive results (out of 20 analyses) were obtained at 200 $\mu\text{g kg}^{-1}$. The detection capability of chlortetracycline was high ($\leq 2000 \mu\text{g kg}^{-1}$), when the manufacturer claimed 600 $\mu\text{g kg}^{-1}$. Some preliminary assays were performed to select the antibiotic concentrations to be validated. Chlortetracycline was tested at 600 and 800 $\mu\text{g kg}^{-1}$. The results were negative. This is the reason why we have decided to validate at 2000 $\mu\text{g kg}^{-1}$. Therefore, the detection capability of chlortetracycline should be included between 1000 and 2000 $\mu\text{g kg}^{-1}$ which was too high regarding the MRL. Microbiological activity profile was quite different for chlortetracycline from oxytetracycline and tetracycline. The detection capability of amoxicillin was very low (5 $\mu\text{g kg}^{-1}$) and lower than the CC β of Explorer® test. This result was satisfactory because no MRL was set for amoxicillin in eggs. Finally, the detection capability for doxycycline was 4 times lower with Premi®Test (200 $\mu\text{g kg}^{-1}$) than with Explorer® test (800 $\mu\text{g kg}^{-1}$). However, considering that this antimicrobial is banned for laying hens, a lower detection capability would have been preferable.

Like for sulphonamides, the detection capabilities of Premi®Test were much lower than those of Explorer® test. One reason could be that egg samples were diluted before analyses with the Explorer® test as it was recommended by the manufacturer.

To our knowledge, only 2 studies have been published on the detection of antimicrobial residues in eggs with Premi®Test (Hussein et al. 2005; Lohajova et al. 2006). Therefore, it was difficult to compare our results with previous studies. Hussein (2005) reported a comparative study between a Four Plate Test (FPT) and Premi®Test for the detection of sulfamethazine (SMZ) in laying eggs after treatment of animals. No false positive results were obtained with both tests. Premi®Test was more sensitive than FPT. The FPT gave positive results when the SMZ concentration was at least equal to 31860 $\mu\text{g kg}^{-1}$. Negative results were observed at 1720 $\mu\text{g kg}^{-1}$. However Premi®Test gave positive results, even at 96 $\mu\text{g kg}^{-1}$ of SMZ (8 days after administration). Finally, high

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

A comparison of STAR microbiological method and Premi®Test for the detection of amoxicillin residues in laying eggs after treatment of animals was also published (Lohajova et al. 2006). Egg samples were extracted before analyses with the STAR protocol, while no extraction was performed before Premi®Test. The conclusion was that Premi®Test was a little more sensitive for amoxicillin than the STAR protocol. In fact, Premi®Test allowed to detect amoxicillin 96h after the end of the animal treatment in egg yolk and 72h in egg white, while the STAR protocol allowed to detect amoxicillin after 72h in egg yolk and 48h in egg white after the end of the treatment. Our validation study also proved that the detection capability of amoxicillin in eggs with Premi®Test was lower than $5 \mu\text{g kg}^{-1}$ which was also the detection limit claimed by the manufacturer.

Cross-reactions.

The percentages (%) of cross-reactivities (CR) were calculated in eggs and were compared with those claimed by the manufacturer for the ELISA kit (RAISIO Diagnostics) (see Table V). The calculated % of cross-reactivities were lower than those given by the manufacturer for sulfadiazine, sulfamerazine and sulfisoxazole. The % CR for sulfachloropyridazine and sulfamethizole are similar. The % CR of cross-reactivities given by the manufacturer were probably calculated in buffer, which could explain the differences.

Insert Table V here

Precision.

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

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2 485 overall coefficients of variation were included between 30 and 40 % (Table VI). It is quite high
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4 486 regarding the criteria of the decision EC/2002/657. However it is acceptable for an ELISA test
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6 487 which is considered as only a semi-quantitative test.
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9 488 Insert Table VI here

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11 489 These results confirmed that the ELISA kit should have been used to study the stability of
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13 490 sulphonamides in eggs because of the high variability of quantitative results.
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15 491
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17 492 *Stability.*
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20 493 As it is required in the decision EC/2002/657 (EC 2002), stability studies should be performed
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22 494 during validation studies. However, the tests implemented in this study were qualitative tests
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24 495 (Premi®Test, Explorer® test) or only semi-quantitative tests (RAISIO Diagnostics ELISA kits). Our
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26 496 experience proved that this kind of tests are not suited to perform stability studies. Therefore,
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28 497 stability data if possible have to be collected in the literature. However, few publications about
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31 498 validation of quantitative methods reported stability data of antimicrobials in food of animal origin,
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33 499 for example after storage at –20°C. Data on the stability of antimicrobials in eggs were much more
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35 500 difficult to find than in more common matrices like milk or muscle. None of the validation studies
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37 501 found in the literature, even for confirmatory methods (physico-chemical), included evaluation of
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39 502 analyte stability for sulphonamides in eggs. Therefore, stability data of sulphonamides and other
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41 503 antimicrobials in milk and tissue samples were presented hereafter when no data was found in
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44 504 eggs.
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46 505 In all studies, the stability of sulphonamide residues in milk at –20°C was proved over 95 days.
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48 506 Juhel-Gaugain (2005) also demonstrated that there was a problem of stability of sulphonamides in
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50 507 tissues during storage at –20°C, particularly in liver. This result confirmed the observations from
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52 508 other studies (Parks et al. 1994). A depletion of the residues at the beginning of the storage at –20
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54 509 °C (during the first month) was observed. Then, sulfa drugs were stable for a long time (Juhel-
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56 510 Gaugain et al. 2005). Sulfamethazine (SMZ) was found stable in bovine milk and piglet muscle
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59 511 samples at –20 and – 75°C during at least 3 and 5 months, respectively (Papapanagiotou et al.
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512 2005). However, a trend of decreasing SMZ levels during prolonged storage at –20°C was clear in
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514 most studies.

The effects of cold storage on the residues of 5 antimicrobials (ampicillin, chloramphenicol, oxytetracycline, streptomycin and sulfamethazine) in meat at +4°C or –20°C were very variable (O'Brien et al. 1981). However, the ampicillin concentration in kidney and meat tissues stored at +4°C or at –20°C decreased. Stability studies showed that penicillin G and oxytetracycline were quite stable in incurred tissue samples. At –20°C, oxytetracycline, chloramphenicol and sulfamethazine seemed to be stable. Moreover, in another study, after 8 months of storage at -20°C, a significant decrease for ampicillin in muscle arose and was never observed at –75°C (Verdon et al. 2000). Some articles have been published about the stability of antimicrobials to heat process (cooking). For example, it was observed that neomycin and streptomycin residues in eggs were quite stable to normal egg cooking procedures (Katz et al. 1978; Inglis et al. 1978). However, there was no information about the stability when eggs were stored at +4°C or –20°C for example. The conclusions on this bibliographical study was that there is a huge lack of data on the stability of antibiotics in eggs. Moreover, when looking at stability studies in other matrices, we could observe a great variability in the results.

Participation to a proficiency test

The results of our lab were satisfactory (Table VII). Firstly because the 2 blank samples were found negative: hen and quail eggs. Secondly, the samples containing sulphonamide residues have been found doubtful (material 4a and 4b (SMZ 50 µg kg⁻¹) and material 5 (SDZ 30 µg kg⁻¹)) or even highly positive (material 3). The great positivity was a logical result because material 3 contained a mix of 2 sulphonamides (SDZ 50 µg kg⁻¹ / SDMX 60 µg kg⁻¹). Therefore, the cumulative concentration was the highest concentration. As a conclusion, the Premi®Test was able to discriminate between negative blank egg samples and egg samples containing sulphonamide residues at levels equal or below 50 µg kg⁻¹.

Conclusion

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

The detection capability is the most important characteristic to be determined for a screening test. The detection capabilities have to be as low as possible for banned substances and lower than MRL when MRLs have been set. The sensitivity of Premi®Test was better than sensitivity of Explorer® test for all the tested sulphonamides and the other tested antimicrobials, probably because of the eggs dilution before Explorer® test. A dilution of egg samples (1/4) before Explorer® analysis could explain that the detection capabilities are too high for most of the antimicrobials. The results of our participation to the proficiency test confirmed the performance of Premi®Test for the detection of sulphonamides in eggs. The detection capabilities for the following antimicrobials were lower than their respective MRLs: amoxicillin, neomycin, tylosin and erythromycin. Therefore, Premi®Test seemed to be more suitable to the wide screening of antimicrobials in eggs as a routine method. Complementary experiments should be implemented to validate Explorer® test and Premi®Test for more antimicrobial residues.

The second characteristic of a screening test should be to minimise the % of false positive results to reduce the number of confirmatory analyses. In this case, the false positive rate was very low for all kits (lower than 2 %) and thus the specificity was satisfactory.

The multi-sulphonamide ELISA kit is of great interest because generally ELISA tests could detect only one antibiotic (Spinks et al. 2001). The CC_β values for sulphonamides (25 to 50 µg kg⁻¹) were much lower than for microbiological tests. This ELISA test should be considered only as a semi-quantitative test because of high coefficient of variation (precision) (between 30 and 40 %). The extraction of egg samples was one disadvantage of ELISA kit. It was more time and money

consuming than microbiological plate tests and also needed a more skilled technician. So this kind of ELISA kit could be recommended for the targeted screening of eggs, for sulphonamides exclusively.

Finally, optical reading and microplate format of Explorer® test were advantages versus visual reading and ampoule format of Premi®Test. High throughput screening could be reach easily. However, optical reading is also possible for Premi®Test with scanner technology (Stead et al. 2005).

As a conclusion, the control of eggs for the presence of antimicrobials could be reinforced with these kind of tests because they are simple, rapid and sensitive enough (Premi®Test and ELISA kit from RAISIO Diagnostics).

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Figure 1. Example of interpretation of quantitative results (Optical Density) with the Explorer® test.

This figure represents one day of validation. The optical densities (OD) (difference between the values of the 2 readings at 2 wavelengths (590 and 650 nm)) are represented on y-axis in relation to the number of analyses on x-axis. The difference of OD of blank samples (negative control NA) are represented with a square shape ■. The difference of OD of positive control (penicillin G at 25 µg kg⁻¹) are represented with a triangle shape ▲. The negative control sample absorbance (NA) + 0.15 is represented with a cross shape ×. The result (SA = Sample Absorbance) of each unknown sample (diamond shape ♦) was compared to this line. A sample was declared positive when: SA 590nm - SA 650 nm ≥ NA590nm - NA 650nm + 0.15.

Figure 2. Determination of detection capabilities for the multi-sulphonamides ELISA kit (RAISIO Diagnostics).

This figure represents the optical densities (OD) (y-axis) in relation to the number of analyses (x-axis). The OD of blank samples are represented with a diamond shape ♦ and the points are linked by a line.

The 40 analyses per sulphonamide' concentration are duplicate analyses of 20 samples over 4 days. The 6 sulphonamides are as follows: S1 = sulfamethazine 25 µg kg⁻¹, S2 = sulfadiazine 50 µg kg⁻¹, S3 = sulfamerazine 25 µg kg⁻¹, S4 = sulfisoxazole 25 µg kg⁻¹, S5 = sulfachloropyridazine 50 µg kg⁻¹ and S6 = sulfamethizole 200 µg kg⁻¹.

Figure 1.

Figure 2.

Deleted: ¶

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

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	/	/

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

Table III. Comparison of the detection capabilities calculated for the 3 kits for sulphonamides and 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.

Table IV. Comparison of the detection capabilities calculated for Premi®Test and Explorer® test for the other antimicrobial residues and comparison with the announced detection limits.

	Premi®Test					Explorer®			
	MRL	Announced LOD (DSM) (µg kg ⁻¹)	Levels of fortification (µg kg ⁻¹)	nb + results /spiked samples	CCβ (µg kg ⁻¹)	Announced LOD (Zeu) (µg kg ⁻¹)	Levels of fortification (µg kg ⁻¹)	nb + results /spiked samples	CCβ (µg 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	/	/	/

LOD: detection limit

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

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

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

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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

Target concentration was 100 µg kg⁻¹.

D2: Day 2 of validation

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

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

CV: Coefficient of variation (%)

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

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

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).

Table VII. Results of our participation to the proficiency test for the detection of sulphonamides in 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

sulfadiazine (SDZ), sulfadimethoxine (SDMX), sulfamethazine (SMZ)

D: Doubtful