Effective screening of antibiotic and coccidiostat residues in food of animal origin by reliable broad-spectrum residue screening tests

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ABSTRACT

Foods of animal origin are controlled for antibiotic and coccidiostat residues. The rapid residue detection is possible using reliable broad-spectrum screening tests. This study’s objective using four microbial inhibition tests for the detection and identification of antibiotic and coccidiostat residues in different foods of animal origin: Premi¹ Test, EXP Ampulle test, Milchtest and Screening Test for Antibiotic Residues (STAR). Four hundred and thirty (430) food samples (165 animal tissues, 152 raw cow’s milk and 113 eggs) were randomly collected and screened. Using the Premi¹ Test, 18 samples were positive and 6 samples dubious. Using the EXP Ampulle test, 31 samples were positive and 2 samples dubious. Using the Milchtest, 15 samples were positive and 12 samples dubious. Using the STAR, 65 samples were positive with 62 samples positive on plates specific for beta-lactams and sulphonamides, 4 samples on plates specific for aminoglycosides, 8 samples on plates specific for macrolides and beta-lactams; and 7 samples on the plates specific for tetracyclines. Retesting using penicillinase and para-aminobenzoic acid (PABA) to confirm the presence of beta-lactams or sulphonamides all potentially positive tube test samples revealed 21 samples positive for beta-lactams and 27 samples positive for sulphonamides. Further testing of sulphonamide positive chicken samples revealed the positivity for coccidiostat salinomycin which was confirmed by testing with PABA, which counteracting salinomycin inhibition. Three hundred and sixty six (366) animal food samples were negative for antibiotic and coccidiostat residues. Microbial inhibition tests are preferred for initial antibiotic screening and have also proven useful for coccidiostat screening and post-screening.

HIGHLIGHTS

- Foodstuffs of animal origin are subject to controls on antibiotic and coccidiostat residues.
- Microbial inhibition tests are still the preferred choice for the initial screening of antibiotic residues in food matrices and could become a useful tool for the screening of coccidiostat residues also.
- More specific post-screening analysis with PABA proved unexpectedly to be a reliable tool in the preliminary detection of coccidiostat residues in poultry meat and eggs.

Introduction

The use of antibiotics or coccidiostats for therapeutic, preventative and/or growth promotion purposes in food-producing animals is causing serious problems associated with the presence of residues in foods of animal origin. The presence of residues in food of animal origin above the maximum levels or residue limits is recognised worldwide as an important food safety and health hazards. The presence of residues in foods may lead to the development of resistant strains of microorganisms, hypersensitivity reactions in sensitive individuals, intestinal microflora disturbances and economic losses in the food industry especially by interfering with starter culture (Kožárová, Mačanga, et al. 2011; Ezenduka et al. 2014; Samandoulougou et al. 2015; Bacanli and Başaran 2019).

Screening of food-producing animals for the presence of antibiotic and coccidiostat residues is one of the main pillars of the European Union (EU) and national monitoring programmes. Commission Decision 2002/657/EC describes a screening method as a method used to detect the presence of a...
The adequate detection of a broad-spectrum of antibiotics is only possible using multi-plate test systems based on a combination of different test organisms (Pikkemaat 2009). In 1999, the Community Reference Laboratory (CRL) in Fougeres (France) developed the Screening Test for Antibiotic Residues (STAR) for the detection of antibiotic residues in milk and meat. With respect to the requirements laid down by the Commission Decision 2002/657/EC, which concerns the performance of analytical methods and the interpretation of results, the STAR method has been validated for antibiotic residue detection in milk (Gaudin et al. 2004), and in meat (Gaudin et al. 2010). The STAR method comprises five test plates sensitive to group or groups of antibiotics as follows: Bacillus subtilis BGA at pH 8.0 for aminoglycosides, Kocuria rhizophila ATCC 9341 at pH 8.0 for macrolides and β-lactams, Bacillus cereus ATCC 11788 at pH 6.0 for tetracyclines, Escherichia coli ATCC 11303 at pH 8.0 for quinolones, and Bacillus steatorrhophilus var. calidolactis ATCC 10149 at pH 7.4 for β-lactams and sulphonamides. The higher specificity and detection capability of this method predetermined the STAR method to be a preferential method for the screening of antibiotic residues in many European countries (Pikkemaat et al. 2008; Kožárová, Mačanga, et al. 2009; Gaudin et al. 2010; Cháfer-Percías et al. 2010; Kožárová, Mačanga, et al. 2011; Gondová and Kožárová 2012; Gondová et al. 2014).

From a practical perspective, commercial tube tests form an attractive alternative to multiplate methods (Pikkemaat 2009). From these tests, the Premi²®Test manufactured and supplied by R-Biopharm AG (Darmstadt, Germany) is the most commonly used today. This test has been validated for the detection of β-lactam residues, cephalosporin, macrolide, tetracycline, sulphonamide, aminoglycoside, quinolone, amphenicol, and polypeptide antibiotics in meat, fish, shrimp, eggs, liver, kidney, plasma/serum, urine and feed in line with EU MRLs (Stead et al. 2004; European Commission 2010). The Premi²®Test contains viable spores of a strain of Bacillus steatorrhophilus var. calidolactis. The use of spores instead of vegetative cells allows prolonged storage and enables commercial distribution. Because of its fast growing properties at elevated temperature, it is possible to obtain a result within a few hours (Pikkemaat et al. 2009).

An attractive alternative to the Premi²®Test are two similar commercially available Bacillus steatorrhophilus tube tests: EXP Ampulle and Milchtest (Packhaus Rockmann GmbH, Sendenhorst, Germany). They have a high degree of sensitivity, thus enabling the...
The samples were stored at 4°C fresh table eggs (organic (12), free-range (18), barn 
Additionally, 152 samples of raw cow ovine animals: meat (2), kidney (2), spleen (2). porcine animals: meat (7), liver (7), kidney (7); spleen (2); bovine animals: meat (12), liver (12), kidney heart (27), gizzard (2), kidney (3), fat and skin (3), spleen (2); bovine animals: meat (12), liver (12), kidney (12); porcine animals: meat (7), liver (7), kidney (7); ovine animals: meat (2), kidney (2), spleen (2)]. Additionally, 152 samples of raw cow’s milk and 113 fresh table eggs (organic (12), free-range (18), barn laid (24), cage (59)] were also included in the study. The samples were stored at 4°C (milk, eggs) and –20°C (meat) until further processing. All samples were taken applying the simple random sampling method. The meat samples were harvested from randomly selected animals slaughtered in a slaughterhouse or purchased at a wholesale market. The chicken kidneys were manually removed from the back of the whole chicken carcasses. The raw cow’s milk samples were taken from on-farm bulk tanks. The egg samples were purchased at the production site or a wholesale market. Several meat samples have been provided to us by the national authority within the monitoring and control of residues.

Screening Test for Antibiotic Residues (STAR)

Preparation of test plates

Test plates were prepared and used according to the STAR protocol Version 3 developed by the Community Reference Laboratory (AFSSA-Fougères, France) adopted by the competent national authority as the officially approved protocol for the screening of products of food-producing animals for antibiotics residues in Slovakia (R-25 2013). Antibiotic medium 11 (Difco 259310; Difco, Detroit, USA) adjusted to pH 8.0 was seeded with ready-to-use commercial spore suspension Bacillus subtilis BGA (5 × 10⁴ spores/mL) (Merck 10649, Merck, Darmstadt, Germany). Test agar pH 8.0 (Merck 10664) was seeded with bacterial suspension Kocuria rhizophila ATCC 9341 (Czech Collection of Microorganisms, Brno, Czech Republic) to give a final concentration of 5 × 10⁴ germs/mL in agar medium. Test agar pH 6.0 (Merck 10663) was seeded with bacterial suspension Bacillus cereus ATCC 11778 (Czech Collection of Microorganisms) to give a final concentration of 3 × 10⁴ germs/mL in the agar medium. Test agar pH 8.0 (Merck 10664) was seeded with bacterial suspension Escherichia coli ATCC 11303 (Czech Collection of Microorganisms) to give a final concentration of 10⁵ germs/mL in the agar medium. Diagnostic Sensitive Test (DST) agar (Oxoid CM 261; Oxoid, Basingstoke, UK) adjusted to pH 7.4 was seeded with ready-to-use commercial spore suspension Bacillus stearothermophilus var. caldololactis ATCC 10149 (5 × 10⁵ spores/mL) (Merck 1.11499) and supplemented with trimethoprim (Fluka 92131; Fluka, Buchs, Switzerland) to obtain a final concentration of 0.005 µg/mL in the agar medium. Finally, 5 ml of the seeded media were poured into Petri dishes of 90 mm in diameter. Commercial agar media were prepared according to the manufacturer’s instructions. Quality control for each test plate was performed using paper discs 9 mm in diameter (Whatman Grade No. 1, Whatman International Ltd, Maidstone, UK) soaked with 30 µL of control standard solutions of reference materials.
antibiotics prepared and stored according to the procedures set by the method.

**Screening of the samples**

**Meat:** A cylindrical core 8 mm in diameter and approximately 2 cm long was removed from each frozen sample using a sterile cork borer (Ø 9 mm) and cut into slices of 2 mm in thickness with a sterile lancet. Cut slices were placed opposite each other on each of the five test plates. **Milk:** A total of 30 µL of raw homogenised cow’s milk was transferred to a filter paper discs 9 mm in diameter (A2668090, Hahnemühle FineArt GmbH, Dassel, Germany), which were placed opposite each other on each of the five test plates. **Eggs:** The egg shell was cracked manually and the content was thoroughly homogenised. Before analysis, all egg samples were pre-incubated in a thermoblock (Acublock Digital Dry Bath D 1200, Labnet, Edison, USA) at 80 °C for 10 min (according to the Premi® Test manufacturer’s instructions.). Eggs were further examined in the same way as milk samples. Test plates were incubated as follows: *Bacillus subtilis* BGA and *Bacillus cereus* ATCC 11778 test plates at 30 °C for at least 18 h, *Kocuria rhizophila* ATCC 9341 and *Escherichia coli* ATCC 11303 test plates at 37 °C for at least 24 h, and *Bacillus stearothermophilus* var. *calidolactis* ATCC 10149 test plates at 55 °C for 12 h.

**Reading the test results**

Samples were considered positive if they gave the inhibition zone (IZ) on at least one of the five test plates either equal or superior to 2 mm wide on plates seeded with *Bacillus subtilis* BGA, *Kocuria rhizophila* ATCC 9341, *Bacillus cereus* ATCC 11778 and *Escherichia coli* ATCC 11303, and equal or superior to 4 mm (meat) or 2 mm (milk, eggs) on plates seeded with *Bacillus stearothermophilus* var. *calidolactis* ATCC 10149. The width of the IZ was measured as the distance between the edge of the slice of the tissue or the disc and the outer limit of the IZ in mm using a digital calliper (Mitutoyo, Kawasaki, Japan) with a precision of 0.01 mm. The diameter of the IZ were expressed as the mean ± standard deviation (SD) of six measures.

**Premi® test**

**Screening of the samples**

Premi®Test was performed according to the officially approved protocol for the determination of residues of inhibitory substances in meat by the Premi®Test (R-26 2013) in Slovakia which is in compliance with the manufacturer’s instructions for use. **Meat:** A total of 100 µL of the juice obtained by thawing the sample in a microwave oven set to defrost were pipetted onto the agar in the ampoule and allowed to stand at room temperature for 20 min for pre-diffusion. After the pre-diffusion, the juice was flushed out of the ampoule by washing twice with demineralised water. For the used kidney and liver samples, ampoules containing 100 µL of kidney and liver juice were pre-incubated at 80 °C for 10 min. After the heat pre-treatment, all ampoules were further tested uniformly by incubation in a digital dry bath (Labnet Accublock Digital Dry Bath D 1200, Labnet, Edison, USA) at 64 °C ± 0.5 °C for approximately 3 to 3.5 h until the negative control turned from purple to yellow. During the incubation period, ampoules were sealed with a plastic foil to avoid evaporation. **Eggs:** A total of 100 µL of the homogenised egg content were pipetted onto the agar in the ampoule. Ampoules were placed into a digital dry bath and pre-incubated at 80 °C for 10 min. After this heat pre-treatment, ampoules were further incubated in the same way as the meat and milk samples as aforementioned. During the incubation period, ampoules were covered with a plastic foil to avoid evaporation.

**EXP Ampule test**

**Screening of the samples**

EXP Ampule test was performed according to the manufacturer’s instructions for use supplied in the test kit (Packhaus Rockmann GmbH, Sendenhorst, Germany). **Meat:** A total of 100 µL of the extracted meat juice obtained by thawing the sample in a microwave oven set to defrost until the sample was fully cooked, were added to the ampoule. Ampoules were incubated at room temperature for 30 min, washed with demineralised water, and excess water was removed by turning the tubes upside down onto the absorbent paper. Ampoules were carefully sealed with an adhesive foil and placed in a digital dry bath incubator at 65 °C ± 1 °C for approximately 3 h and 15 min until the negative control turned from purple to yellow. **Eggs:** 10 mL of sterile demineralised water were added to 10 mL of the homogenised egg content. After thorough mixing, the diluted sample was warmed up for 3 min in a water bath at 100 °C with occasional mixing with a glass rod to prevent coagulation. A 100 µL of the diluted homogenised sample were transferred to the ampoule. The ampoule was covered with an adhesive foil and incubated in the same way as the meat samples.
**Milchtest**

**Screening of the samples**

Milchtest was performed according to the manufacturer's instructions for use supplied in the test kit (Packhaus Rockmann GmbH, Sendenhorst, Germany). A total of 50 μL of the milk sample were transferred to the ampoule. The ampoule was sealed with a foil supplied with the kit and transferred to a digital dry bath incubator where the sample was incubated for about 3 h at a temperature of 65 ± 0.5 °C. The test was terminated when the colour of the agar medium of the negative control changed from violet to yellow.

**Reading the results of the Premi<sup>V</sup>R test, EXP Ampulle test and Milchtest**

The test was terminated when the negative control sample turned yellow. A clear colour change from purple to yellow indicated that the sample contained no antibiotic residues or that the concentration of residues was below the detection limit of the respective test. When the colour remained purple or the colour of the sample was clearly different to that of the negative control, the sample contained antibiotic residues at a concentration above the detection limit of the respective test and were considered dubious. The test results were evaluated using a colour card or a colour scale supplied by the manufacturer with the kit.

**Post-screening confirmation of beta-lactams and sulphonamides**

**Identification of beta-lactam antibiotics**

A total of 100 μL of penicillinase (Penase, SR0129, Oxoid, Basingstoke, UK, 50 IU/mL) was pipetted onto agar in test tubes and allowed to stand at room temperature for 30 min for pre-diffusion. After pre-diffusion, penicillinase was flushed away from the ampoules and the sample was further tested using the Premi<sup>V</sup>R Test, EXP Ampulle test and Milchtest screening procedures mentioned above. If a previously positive sample on screening gave a negative result by a colour change of the medium from purple to yellow, it confirmed an indication of the presence of beta-lactam antibiotics in the sample.

**Identification of sulphonamides**

PABA stock solution (A 9878, Sigma-Aldrich, St. Louis, USA; 1000 μg/mL) was prepared by dissolving 10 mg PABA powder in 10 mL sterile demineralised water. 100 μL of working PABA solution at the concentration of 100 μg/mL was placed onto the agar in respective ampoules and allowed to stand at room temperature for 30 min for pre-diffusion. After pre-diffusion, PABA was flushed away from the ampoules and the sample was further tested using the Premi<sup>V</sup>R Test, EXP Ampulle test and Milchtest as mentioned above. When a previously positive screened sample gave a negative result presented by a colour change of the medium from purple to yellow, it confirmed the presence of sulphonamides in the sample.

**Statistical analysis**

The Chi-square test was performed to compare the data of the Premi<sup>V</sup>R Test and the EXP Ampulle test used for the screening of the same animal food samples with a degree of freedom 4. The significance level was 0.05. The statistical analysis was performed using the online tool [http://www.quantpsy.org/chisq/chisq.htm](http://www.quantpsy.org/chisq/chisq.htm).

**Results**

Screening results for the presence of antibiotic and coccidiostat residues using four microbial inhibition tests are presented in Table 1. A total of 430 samples subjected to residues analysis by inhibition of growth of sensitive bacterial strains, 65 samples (15.12%) yielded a positive or dubious result on one or more screening tests.

Residue screening using commercial tube tests containing *Bacillus stearothermophilus* var. *calidolactis* as the indicator organism revealed that 18 samples (4.19%) of samples including 6 chicken livers, 2 hearts, 3 fats/skins, 2 spleens, 2 gizzards; 1 porcine muscle and 2 eggs were positive and 6 samples (1.40%) including 3 chicken livers and 3 eggs were dubious on the Premi<sup>V</sup>R Test, On the EXP Ampulle test, 31 samples (7.21%) including 9 chicken livers, 1 heart, 1 kidneys, 3 fats/skins, 2 spleens, 2 gizzards and 13 eggs were positive and 2 samples (0.47%) including 1 chicken liver and 1 kidneys dubious. On the Milchtest, 15 milk samples (3.49%) were positive and 12 milk samples (2.79%) dubious. Applying the Chi-square statistics to the results of the Premi<sup>V</sup>R Test and the EXP Ampulle tube tests, we found that the p-value was .6590 (p > .05) with the Yates of correction 0.8. This indicates that the two tests do not exhibit any significant differences.

On the STAR method, 62 samples (14.42%) including 10 chicken livers, 2 kidneys, 2 hearts 3 fats/skins, 2 spleens, 2 gizzards; 1 porcine muscle, 13 egg and 27...
Table 1. Overview of the positive results obtained from the screening of animal food samples for the presence of antibiotic and coccidiostat residues using the STAR, Premi\textsuperscript{PR}V Test, EXP Ampulle and Milchtest.

| Animal Matrix | Bacillus subtilis ATCC 9341 | Bacillus cereus ATCC 11778 | Escherichia coli ATCC 11303 | Bacillus stearothermophilus var. caldolactis ATCC 10149 | STAR | Premier\textsuperscript{PR}V Test | EXP Ampulle | Milchtest |
|---------------|-----------------------------|-----------------------------|-----------------------------|----------------------------------------------------------|-----|-----------------------------|-------------|-----------|
| Chicken Liver | 5.16 ± 0.52                 | ±                          | +                           |                                             | 28/10\textsuperscript{^3} | 35.71\%\textsuperscript{^5} |                       |           |
| Chicken Liver | 4.36 ± 0.22                 | −                          | +                           |                                             | 28/9\textsuperscript{^3} | 32.14\%\textsuperscript{^5} |                       |           |
| Chicken Liver | 4.04 ± 0.29                 | +                          | +                           |                                             | 28/10\textsuperscript{^3} | 35.71\%\textsuperscript{^5} |                       |           |
| Chicken Liver | 4.04 ± 0.45                 | +                          | +                           |                                             | 28/9\textsuperscript{^3} | 32.14\%\textsuperscript{^5} |                       |           |
| Chicken Liver | 4.37 ± 0.34                 | +                          | +                           |                                             | 28/10\textsuperscript{^3} | 35.71\%\textsuperscript{^5} |                       |           |
| Chicken Liver | 4.64 ± 0.45                 | +                          | −                           |                                             | 28/9\textsuperscript{^3} | 32.14\%\textsuperscript{^5} |                       |           |
| Chicken Liver | 5.65 ± 0.33                 | +                          | +                           |                                             | 28/10\textsuperscript{^3} | 35.71\%\textsuperscript{^5} |                       |           |
| Chicken Liver | 11.38 ± 0.43                | ±                          | +                           |                                             | 28/9\textsuperscript{^3} | 32.14\%\textsuperscript{^5} |                       |           |
| Chicken Liver | 6.88 ± 2.44                 | −                          | −                           |                                             |                       |                               |                   |           |
| Kidney Liver  | 6.36 ± 0.82                 | −                          | +                           |                                             |                       |                               |                   |           |
| Kidney Liver  | 6.30 ± 1.23                 | −                          | −                           |                                             |                       |                               |                   |           |
| Kidney Liver  | 7.71 ± 0.44                 | −                          | −                           |                                             |                       |                               |                   |           |
| Muscle Liver  | 5.14 ± 0.53                 | −                          | −                           |                                             |                       |                               |                   |           |
| Muscle Liver  | 2.37 ± 0.61                 | −                          | −                           |                                             |                       |                               |                   |           |
| Heart Liver   | 5.45 ± 0.78                 | +                          | +                           |                                             | 37/2\textsuperscript{^5} | 5.41\%\textsuperscript{^5} |                       |           |
| Heart Liver   | 5.91 ± 0.67                 | +                          | +                           |                                             | 37/2\textsuperscript{^5} | 5.41\%\textsuperscript{^5} |                       |           |
| Heart Liver   | 27/2\textsuperscript{^5}    | 7.41\%\textsuperscript{^5} | 3.70\%\textsuperscript{^5} |                                             |                       |                               |                   |           |
| Fat/skin Liver| 4.15 ± 0.19                 | +                          | +                           |                                             | 3/3\textsuperscript{^5} | 100\%\textsuperscript{^5} |                       |           |
| Fat/skin Liver| 5.54 ± 0.48                 | +                          | +                           |                                             | 3/3\textsuperscript{^5} | 100\%\textsuperscript{^5} |                       |           |
| Fat/skin Liver| 5.50 ± 0.34                 | +                          | +                           |                                             | 3/3\textsuperscript{^5} | 100\%\textsuperscript{^5} |                       |           |
| Spleen Muscle | 11.05 ± 0.71                | ±                          | +                           |                                             | 2/2\textsuperscript{^5} | 100\%\textsuperscript{^5} |                       |           |
| Spleen Muscle | 12.22 ± 0.12                | ±                          | +                           |                                             | 2/2\textsuperscript{^5} | 100\%\textsuperscript{^5} |                       |           |
| Gizzard Muscle | 4.11 ± 0.40               | 4.01 ± 0.20                | 3/3\textsuperscript{^5} |                                             |                       |                               |                   |           |
| Gizzard Muscle | 4.01 ± 0.20               | 4.01 ± 0.20                | 3/3\textsuperscript{^5} |                                             |                       |                               |                   |           |
| Porcine Muscle | 4.51 ± 0.54               | 7/1\textsuperscript{^5}    | 14.29\%\textsuperscript{^5} |                                             |                       |                               |                   |           |
| Bovine Milk   | 2.19 ± 0.97                 | ±                          | +                           |                                             |                       |                               |                   |           |
| Bovine Milk   | 3.43 ± 0.59                 | 3.43 ± 0.59                | ±                            |                                             |                       |                               |                   |           |
| Bovine Milk   | 4.66 ± 0.23                 | 4.66 ± 0.23                | ±                            |                                             |                       |                               |                   |           |
| Bovine Milk   | 5.33 ± 0.77                 | 5.33 ± 0.77                | ±                            |                                             |                       |                               |                   |           |
| Bovine Milk   | 5.47 ± 0.30                 | 5.47 ± 0.30                | ±                            |                                             |                       |                               |                   |           |
| Bovine Milk   | 4.53 ± 0.30                 | 4.53 ± 0.30                | ±                            |                                             |                       |                               |                   |           |
| Bovine Milk   | 5.98 ± 0.82                 | 5.98 ± 0.82                | ±                            |                                             |                       |                               |                   |           |
| Bovine Milk   | 2.23 ± 0.16                 | 2.23 ± 0.16                | ±                            |                                             |                       |                               |                   |           |
| Bovine Milk   | 4.75 ± 0.31                 | 4.75 ± 0.31                | ±                            |                                             |                       |                               |                   |           |
| Bovine Milk   | 4.87 ± 0.33                 | 4.87 ± 0.33                | ±                            |                                             |                       |                               |                   |           |
| Bovine Milk   | 5.89 ± 0.66                 | 5.89 ± 0.66                | ±                            |                                             |                       |                               |                   |           |
| Bovine Milk   | 6.81 ± 2.09                 | 6.81 ± 2.09                | ±                            |                                             |                       |                               |                   |           |
| Bovine Milk   | 6.82 ± 0.69                 | 6.82 ± 0.69                | ±                            |                                             |                       |                               |                   |           |
| Bovine Milk   | 4.15 ± 0.47                 | 4.15 ± 0.47                | ±                            |                                             |                       |                               |                   |           |
| Bovine Milk   | 3.91 ± 0.18                 | 3.91 ± 0.18                | ±                            |                                             |                       |                               |                   |           |
| Bovine Milk   | 3.38 ± 0.78                 | 3.38 ± 0.78                | ±                            |                                             |                       |                               |                   |           |
| Bovine Milk   | 2.00 ± 0.17                 | 2.00 ± 0.17                | ±                            |                                             |                       |                               |                   |           |
| Bovine Milk   | 4.15 ± 1.12                 | 4.15 ± 1.12                | ±                            |                                             |                       |                               |                   |           |
| Bovine Milk   | 5.83 ± 2.47                 | 5.83 ± 2.47                | ±                            |                                             |                       |                               |                   |           |
| Bovine Milk   | 5.12 ± 1.18                 | 5.12 ± 1.18                | ±                            |                                             |                       |                               |                   |           |
| Bovine Milk   | 4.31 ± 0.64                 | 4.31 ± 0.64                | ±                            |                                             |                       |                               |                   |           |
| Bovine Milk   | 4.12 ± 0.19                 | 4.12 ± 0.19                | ±                            |                                             |                       |                               |                   |           |
| Bovine Milk   | 1.63 ± 1.43                 | 1.63 ± 1.43                | ±                            |                                             |                       |                               |                   |           |
| Bovine Milk   | 2.04 ± 0.62                 | 2.04 ± 0.62                | ±                            |                                             |                       |                               |                   |           |
| Bovine Milk   | 3.98 ± 0.73                 | 3.98 ± 0.73                | ±                            |                                             |                       |                               |                   |           |
milk samples were positive on the Bacillus stearothermophilus var. calidolactis ATCC 10149 plates specific for beta-lactams and sulphonamides, 4 samples (0.93%) including 1 chicken kidneys, 2 muscles and 1 milk sample were positive on the Bacillus subtilis BGA test plates specific for aminoglycosides, 8 samples (1.86%) including egg samples were positive on the Kocuria rhizophila ATCC 9341 test plates specific for macrolides and beta-lactams and 7 samples (1.63%) including milk samples were positive on the Bacillus cereus ATCC 11778 test plates specific for tetracyclines. No inhibition zones were observed on the Escherichia coli ATCC 11303 test plates specific for quinolones.

Among all positive food samples, milk and egg samples showed the formation of inhibition zones on more test plates as follows: 7 milk samples on Bacillus stearothermophilus var. calidolactis ATCC 10149 and Bacillus cereus ATCC 11778 test plates, 1 milk sample on the Bacillus stearothermophilus var. calidolactis ATCC 10149, Bacillus cereus ATCC 11778 and Bacillus subtilis BGA test plates, and finally, 8 egg samples on the Bacillus stearothermophilus var. calidolactis ATCC 10149 and Kocuria rhizophila ATCC 9341 test plates. Based on the sensitivity of the STAR test strains, the positive samples were deemed suspect for the presence of beta-lactams or sulphonamides followed by macrolides, tetracyclines and aminoglycosides, respectively.

Summarising the results of all positive results of all tube tests and the STAR method, 62 samples were positive (13 of which were dubious) by at least one tube test and 65 samples were positive as determined by the STAR method. The additional post-screening analysis of beta-lactams or sulphonamides in the screen-positive samples using penicillinase and PABA revealed the following: 1 porcine muscle sample and 20 milk samples were positive for beta-lactam antibiotics; and all chicken livers (10), 2 kidneys, 2 hearts, 3 fats/skins, 2 spleens and 2 gizzards and 5 eggs were positive for sulphonamides (Figure 1). Based on the screening and post-screening results obtained, we can conclude that positive chicken livers, heart, fats/skins, spleens and gizzards are suspected for the presence of sulphonamides, chicken kidneys for the presence of sulphonamides and aminoglycosides, chicken muscle for the presence of aminoglycosides, porcine muscle for the presence of beta-lactams, egg samples for the presence of sulphonamides and macrolides, and milk samples for the presence of beta-lactams and tetracyclines.

Having the muscles and giblets available from two partially eviscerated broiler chicken carcasses fed
commercially-produced feed containing 70 mg/kg of salinomycin in complete feed, this led us to question whether these chicken samples would prove positive for the coccidiostat salinomycin. The samples from both of these carcasses were subjected to chemical confirmation for coccidiostat residues by using the liquid chromatography (LC) coupled with electrospray ionisation tandem mass spectrometry (MS/MS) according to the procedure reported by Tkáčiková et al. (2010, 2012). Analysis by LC-MS/MS confirmed the presence of salinomycin residues in the chicken liver (8.42 ± 1.94 μg/kg), kidneys (31.2 ± 7.18 μg/kg), fats/skin (76.3 ± 17.55 μg/kg), heart (11.83 ± 2.72 μg/kg), gizzard (30.31 ± 6.97 μg/kg) and also in the muscle (3.33 ± 0.77 μg/kg).

Taking into account the outcomes of the confirmatory analysis determining the presence of salinomycin in the examined chicken tissue samples and given the sulphonamide positive results after post-screening analysis with PABA, the effect of PABA on salinomycin was evaluated by testing the working solutions of salinomycin standard (S 46729, Sigma-Aldrich, St. Louis, USA) at the concentration of 50 and 100 μg/L. The stock solution of the salinomycin standard was prepared by dissolving 10 mg of salinomycin in 1 ml of 5% methanol (Merck, Darmstadt, Germany) and supplemented with sterile demineralised water to a concentration of 1000 μg/L. The working solutions of salinomycin were prepared by serial dilutions with sterile demineralised water to the final concentration of 50 μg/L. A total of 100 μL of respective salinomycin working solutions were transferred to the ampoules of the Premi® Test and EXP Ampulle test previously fortified with PABA according to the procedure used for
the post-screening identification of sulphonamides and further tested using both tube tests screening procedures mentioned above. To investigate the detection capability of the Premi® Test, EXP Ampulle test and the STAR method, the stock solution was further diluted with sterile demineralised water to reach the concentration of 10 μg/L in view of the MRL currently established for salinomycin in liver (150 μg/kg), kidney (40 μg/kg), muscle (15 μg/kg) and skin/fat (150 μg/kg) of chickens reared for fattening and chickens reared for laying by the Commission Implementing Regulation (EU) 2017/1914 (European Commission 2014).

PABA completely reversed the inhibitory activity of salinomycin at the concentration of 50 and 100 μg/L on the test organism Bacillus stearothermophilus var. calidolactis by visible decolourisation of the agar medium from purple to yellow and thereby confirmed the presence of salinomycin in the positive chicken tissues. This means that PABA seems to be a suitable neutralisation solution for presumptive identification of salinomycin residues in screening-positive samples (Figure 1). Therefore, 5 eggs detected positive for sulphonamides could also be positive for salinomycin. Based on the code of the originating farm stamped on these eggs we found that all these eggs were paradoxically organic eggs produced in country in which coccidiostats are still approved for laying hens.

By testing the salinomycin standard solutions with the Premi® Test, EXP Ampulle test and the STAR method, the test organism Bacillus stearothermophilus var. calidolactis (in the STAR method Bacillus stearothermophilus var. calidolactis ATCC 10149) appeared to be sensitive to salinomycin at the level of the concern. The detection capability of the Premi® Test, EXP Ampulle test and the STAR method for salinomycin was as follows: Premi® Test 10 μg/L, EXP Ampulle test 50 μg/L (10 and 20 μg/L dubious) and STAR 75 μg/L. Salinomycin also inhibited the growth and multiplication of the test organism Bacillus cereus ATCC 11778 of the STAR method. However, the detection capability of this test organism for salinomycin was 500 μg/L. A comparison of the detection capability of the Premi® Test, EXP Ampulle test and the STAR method for salinomycin is presented in Table 2.

Discussion

A wide range of antimicrobial substances with different mechanisms of action are being used in food-producing animals as therapeutic agents to treat diseases or as preventive agents when diseases cannot be eliminated by other means. The endangering of public health associated with the administration of these substances to food-producing animals has increased the need to monitor the residues of these substances in live animals and animal products. The measures to monitor certain substances and residues in live animals and animal products are governed by Council Directive 96/23/EC. These substances are included in Group A as substances having anabolic effect and unauthorised substances and in Group B as veterinary drugs and contaminants. Veterinary drugs are further divided into Group B1 as antibacterials (beta-lactams, tetracyclines, macrolides, aminoglycosides, sulphonamides, quinolones) and Group B2 as other veterinary drugs/B2a/, sedatives/B2d/, non-steroidal anti-inflammatory drugs/B2e/, and other pharmacologically active

Table 2. Comparison of the detection capability of the STAR, Premi® Test and EXP Ampulle test for salinomycin determined by testing the working solutions of salinomycin standard.

| MRL for salinomycin (μg/kg) | Working solution of salinomycin standard (μg/L) | Bacillus subtilis ATCC 9341 | Kocuria rhizophila ATCC 10149 | Bacillus cereus ATCC 11778 | Escherichia coli ATCC 11303 | Bacillus stearothermophilus var. calidolactis ATCC 10149 |
|-----------------------------|-----------------------------------------------|-----------------------------|----------------------------|-----------------------------|------------------------------------------------|-------------------------------------------------|
| Liver (150)                 | 500                                           | 0                          | 0                          | 1.40 ± 0.15                 | 0                                             | 7.68 ± 1.23                                    |
| Kidney (40)                 | 100                                           | 0                          | 0                          | 0                           | 0                                             | 6.97 ± 0.44                                    |
| Muscle (15)                 | 75                                            | 0                          | 0                          | 0                           | 0                                             | 2.51 ± 0.34                                    |
| Skin/Fat (150)              | 50                                            | 0                          | 0                          | 0                           | 0                                             | 0                                              |
|                            | 20                                            | 0                          | 0                          | 0                           | 0                                             | 0                                              |
|                            | 10                                            | 0                          | 0                          | 0                           | 0                                             | ±                                              |

MRL: Maximum Residue Limit.
STAR: Screening Test for Antibiotic Residues.
SD: standard deviation.
+: positive results; –: negative results; ±: dubious results.
substances/B2f/) (Council of the European Union 1996; Kožárová 2018).

It is important to mention that in some EU Member States there are specific control programmes which use microbiological tests (inhibitor tests). In some cases, a positive result in a microbiological test is sufficient to reject the sample. This may mean that no confirmation by a physico-chemical method is carried out and thus there is no conclusive identification of the substance concerned. In other cases, a positive result in the screening test is confirmed by means of an immunochemical or physico-chemical test and it is then possible to identify the substance and establish whether its concentration is above the MRL or not (European Food Safety Authority 2019).

The use of the microbial screening tests or methods within the residue detection is a very cost-effective way of reducing the number of samples that need to be analysed with an expensive physico-chemical confirmation. Another important advantage, compared for example to the confirmatory LC-MS systems, is that the microbial inhibition tests can detect any antibiotic or metabolite with antibacterial activity, whereas LC-MS systems are commonly applied to the compounds previously selected as targets, so that any other antibiotics present would pass undetected (Picó and Barceló 2008; Cháfer-Pericás et al. 2010).

There is a whole range of such tests or methods with various test organisms used worldwide. Their development dates back to the second half of the 20th century and in recent years, significant progress has been seen in this area. The current tests or methods have the capability for a high sample throughput and are used to sift large numbers of samples for the potential non-compliant results. The suitable selection of a respective microbial inhibition test or the use of appropriate tube and plate test combination models constitutes an efficient mean of controlling of a wide range of antimicrobial residues in animal products and foodstuffs. A feasible post-screening confirmatory test using a substance which selectively reserves the inhibitory activity of a respective class of antimicrobials significantly reduces the efforts devoted to the identification and quantitation of the residue by the physico-chemical methods and generates considerable savings of time and resources (Myllyniemi 2004; Pikkemaat 2009; Cháfer-Pericás et al. 2010; Sanz et al. 2015).

Many authors, based on the evaluation of the sensitivity and specificity of microbial inhibition tests, confirmed their suitability and practical applicability for the screening of antimicrobial residues in foods of animal origin at the first stage of the residue monitoring and control strategy. A few examples are given below. Pikkemaat et al. (2009, 2011), in two similar studies, evaluated and compared the performance of a commercial tube Premi®Test and three multi-plate tests, the Four-Plate Test (FPT; Bogaerts and Wolf 1980), the STAR method and the Nouws antibiotic test (NAT; Pikkemaat et al. 2008) for the screening of antimicrobial residues in meat and kidneys of slaughter animals taken as the part of the national monitoring programme. The authors found that the FPT lacks sufficient sensitivity to be used in the routine monitoring, the Premi®Test showed a slightly better result; however, it exhibits a very high false-positive rate and the STAR method and the NAT appeared to be the most sensitive tests; this reduces the confirmatory efforts. The number of suspect samples detectable by the STAR method and the NAT were comparable. Based on the antibiotic group identification, the samples tested positive were deemed suspect for the presence of tetracyclines, sulphonamides, macrolides, aminoglycosides and beta-lactams. In all samples for which the presence of residues were confirmed, the antibiotic group identification using the STAR method and the NAT appeared correct. In both studies, the suspect samples were always re-tested in the presence and absence of penicillinase for the confirmation of the presence of beta-lactam antibiotics.

The incidence of antimicrobial residues in market muscle samples from different animal species (bovine, ovine, porcine and poultry) was evaluated with a screening strategy that combined two other commercial tube tests, a broad spectrum test Explorer (Zeulab, Zaragoza, Spain) and a specific test for quinolones detection Equinox (Zeulab, Zaragoza, Spain) by Sanz et al. (2015). The authors declared that a combination of the Explorer and the Equinox tests appears to be a useful tool since it would enable a broad screening of antimicrobials, especially quinolones, in the muscle samples. The supplementary tests performed to obtain additional information about the nature of antimicrobials in positive muscle samples confirmed the presence of tetracyclines, aminoglycosides, sulphonamides and quinolones. Similar results were obtained by Gaudin, Hedou, Rault, and Verdon (2009). They reported that the Explorer was able to detect compounds belonging to different antimicrobial families (penicillins, cephalosporins, tetracyclines, sulphonamides and macrolides) in the muscle samples from different species (bovine, porcine, ovine and poultry) with the detection capabilities around the MRL level for the tested antimicrobials.
Gaudin, Hedou, Rault, Sanders, et al. (2009) compared the Explorer and the Premi® Test for the detection of antimicrobial and sulphonamide residues in eggs. The sensitivity of the Premi® Test was better than that of Explorer test for all the tested sulphonamides and the other tested antimicrobials, probably because of the dilution of the eggs before Explorer test, as recommended by the manufacturer. In spite of this finding, the authors recommended to use both tests as the wide screening tests allowing the detection of most of the antimicrobial families in eggs. El Nasri et al. (2012) used the disc assay method with Bacillus stearothermophilus (Fagbamila et al. 2010) and the Premi® Test for the detection of antibiotic residues in table eggs. They showed a poor hygiene status of the poultry farms with the concomitant use of antibiotics for the treatment of diseases and the lack of the knowledge regarding the use of antibiotics resulted in the high percentage of positive samples. The Premi® Test detected lower positive percentage of the positive samples while the disc assay correlated well with this excessive use of antibiotics. Shahbazi et al. (2016) assessed the prevalence of drug residues in eggs using the FPT. According to the results of this study, the highest contamination rate of antibiotic residues was related to penicillin and tetracycline groups following aminoglycosides.

There are also a lot of studies evaluating the Bacillus stearothermophilus based tube tests for the routine screening of antibiotic residues in cow, sheep and goat milk (Navrátilová 2009; Pikkemaat 2009; Sierra et al. 2009; Sýkorová Goffová et al. 2012; Wu, Zhu, et al. 2019). In the case of the milk, the primary control begins on the farm and all food business operators must initiate procedures to ensure that raw milk is not placed on the market if it contains antibiotic residues above the level of EU MRL (European Commission 2004). Recently, Wu, Peng, et al. (2019) introduced a novel broad-spectrum microbiological inhibition method for the rapid screening of different kinds of antibiotics such as β-lactam, aminoglycosides, tetracyclines, sulphonamides, macrolides, lincosamides and quinolones in milk, chicken egg and honey by using the microbiological system in microtiter plates with test bacteria Geobacillus stearothermophilus var. C 953. It was observed that the limit of the detection of the kit used in this study for all kinds of antibiotics in milk was lower than or close to the maximum residue limits determined by EU. For chicken egg and honey, the detection capability of the kit was similar to that determined in milk. Moreover, it was revealed that the kit in the present study was more sensitive to aminoglycosides, macrolides and quinolones in various matrices than internationally available commercial kits.

In spite of the fact that the microbial inhibition tests or methods are preferential methods for the screening of antimicrobial residues in foods of animal origin, many authors are convinced that there is still a need for rapid screening methods with a wider spectrum of detection and improved detection capabilities.

All the microbial inhibition tests used in our study characterised the samples as positive or negative. Evaluation of the results of the Premi® Test and the EXP Ampulle tube tests based on the presence or absence of colour change, both tests yielded a comparable number of the positive/dubious results except for 2 chicken kidneys and table eggs mainly in which a higher number of positive results were detected by the EXP Ampulle test. The most positive/dubious results were found in the chicken tissues and eggs. All bovine, ovine and porcine tissue samples with exception of 1 porcine muscle sample yielded negative results. From all chicken matrices examined, the most positive results were found in chicken livers, followed by fats/skins, spleens, gizzards, heart, and finally, kidneys. No positive results were detected in chicken muscles.

Evaluating the results of the STAR method based on the formation of the inhibition zone around the animal post-slaughter matrices exceeding 2 or 4 mm in width dependent on the test organism, the most positive results were found also in chicken matrices. From all chicken matrices, the largest inhibition zones were detected around the chicken spleens followed by livers, kidneys, hearts, fats/skins, gizzards, and finally, muscle (2.37 ± 0.61 mm – 12.22 ± 0.12 mm). No inhibition zones were detected around the bovine, ovine and porcine tissue samples with exception of 1 porcine muscle sample, in which the size of the inhibition zone was 4.51 ± 0.54 mm. Evaluating the results of the positive egg and milk samples, the mean diameters of the inhibition zones ranged from 4.29 ± 0.42 to 15.73 ± 0.48 mm and 2.19 ± 0.97 to 6.82 ± 0.69 mm, respectively.

The positive samples were deemed suspect for the presence of β-lactams or sulphonamides followed by macrolides, tetracyclines and aminoglycosides, respectively. As the highest number of positive results were detected with the Bacillus stearothermophilus var. calidolactis ATCC 10149 test organism very sensitive to β-lactam antibiotics and sulphonamides, it was deemed necessary to perform the post-screening confirmatory analysis of all these positive samples to make a distinction between β-lactam antibiotics.
and sulphonamides by using all tube tests with the use of penicillinase and PABA. The post-screening analysis found a clear distinction between both main classes of antimicrobial substances: beta-lactam antibiotics and sulphonamides; with sulphonamides confirmed only in chicken tissues and eggs.

Taking into account the outcomes of the confirmatory analysis determining the presence of salinomycin in the examined chicken tissue samples and given the sulphonamide positive results after post-screening analysis with PABA, this led us to conduct further analysis to verify whether PABA could actually block the action of salinomycin. Salinomycin is a monocarboxylic polyether ionophore used as an anticoccidial agent in the poultry industries. Salinomycin also exhibits antibacterial activity, especially against Gram-positive bacteria, including various antibiotic-resistant species of Streptomyces. Antifungal, antiparasitic, antiviral, anti-inflammatory, and most recently, anticancer activities have also been reported for salinomycin (Dewangan et al. 2017). Our results confirmed the antibacterial activity of salinomycin on Gram-positive bacteria (Bacillus stearothermophilus var. calidolactis, Bacillus cereus ATCC 11778) used as the test organisms of the microbial inhibition tests, which are used as the main method for the screening of antibiotic residues in products of animal origin. Interestingly the results also revealed the unexpected finding that PABA antagonises the antibacterial action of salinomycin. As, until now, there is no existing literature on this fact and no other studies are published for comparison of our results in this subject area, further experimental studies are needed to build on this finding, to understand the principle of the specific interaction between salinomycin and PABA and to verify the effect of PABA on the antibacterial (anticoccidial) activity of the other coccidiostats authorised for food-producing animals.

The residue control in the European Union pursues the primary goal to protect consumers from intolerable health hazards which may be associated with the residues of veterinary drugs or non-licensed or forbidden substances in animal products (Sterk 2015). The latest European Food Safety Authority (EFSA) report summarises the monitoring data collected from EU Member States in 2017 (European Food Safety Authority 2019). Overall, the percentage of non-compliant samples in 2017 (0.35%) was comparable to the previous 10 years (0.25%–0.37%). 109,260 targeted samples were analysed for substances in the group B1 (antibacterials) of which 284 samples (0.26%) were non-compliant. 111,029 targeted samples were analysed for substances in the group B2 (other veterinary drugs) of which 182 samples (0.16%) were non-compliant. Of a total of samples analysed for substances in the Group B2, 33,151 targeted samples were analysed for substances in the subgroup B2b (anticoccidials) of which 49 samples (0.15%) were non-compliant. Non-compliant samples for antibacterials were reported in bovines (0.31%), ovine/caprine (0.24%), swine (0.3%), poultry (0.1%), milk (0.18%), equine (0.39%), rabbits (0.44%) and eggs (0.25%). Non-compliant samples for anticoccidials were reported in equine (0.85%), swine (0.01%), poultry (0.21%), rabbits (0.65%) and eggs (0.47%). Salinomycin was e.g. detected in poultry (12), eggs (2) and rabbits (2). Since 2009, an important decrease has been observed in the frequency of non-compliant samples for anticoccidials in poultry. This decrease is most likely the result of increased awareness and the measures that followed as a result of the implementation of the Commission Directive 2009/8/EC which set up maximum levels of unavoidable carry-over of coccidiostats in non-target feed (European Commission 2009).

The EU requires by law that foodstuffs such as meat, milk or eggs must not contain residue levels of veterinary medicines or biocidal products that might present a hazard to the health of the consumer (European Medicines Agency 2019). The results of the monitoring of residues of antimicrobial substances in live animals and animal products submitted by the Member States to the European Commission every year, the results of research papers and scientific studies published by authors in this field, and finally, the results of our study clearly indicate that monitoring and control of residues of antibiotics and coccidiostats are still necessary in order to verify the safety of products of animal origin and to protect the public health.

Conclusions

Residue control is very important to ensure food safety. Microbial inhibition tests are methods of choice for in the initial screening of antibiotic residues in food of animal origin. In spite of the fact that microbial inhibition tests do not provide the data on sensitivity to coccidiostats, the antibacterial activity of salinomycin allows us to confirm our previous findings and contribute additional evidence to the fact that the microbial inhibition tests which use the test organism Bacillus stearothermophilus var. calidolactis as their test organism are suitable for the effective detection of salinomycin residues in food of animal origin. The spectrum of antimicrobials detectable by the screening methods described is not exhaustive and there is still
scope for its further expansion and improvement. The outcomes of our study may also be promising with respect to the unexpected finding that PABA counteracted the inhibition of salinomycin on the test organism Bacillus stearothermophilus var. calidolactis. This means that in addition to beta-lactams and sulphonamides, the presence of coccidiostats should also be taken into account. The two-tier testing system based on the microbial screening and more specific post-screening is proven to be an effective tool in controlling residues of antimicrobial substances in food of animal origin and, equally, is an effective tool in the initial screening of coccidiostat residues in poultry meat and eggs.

**Ethics statement**

This study does not contain any studies with animals performed by the authors. All procedures used for the sample collection were routine non-invasive procedures.

**Disclosure statement**

The authors declare that there is no conflict of interest associated with the paper.

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