Comparison of four microbiological inhibition tests for the screening of antimicrobial residues in the tissues of food-producing animals

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Abstract

The study compares two existing microbiological inhibition tests, Screening Test for Antibiotic Residues (STAR) and Premi®Test with two recently introduced tests, Nouws Antibiotic Test (NAT) and Total Antibiotics, Premi® Test, NAT. The growth inhibition was observed in three out of seven test strains, namely Bacillus cereus ATCC 11778, Kocuria rhizophila ATCC 9341, and Bacillus stearothermophilus var. calidolactis. Considering the test strains sensitivity and no inhibition on the Bacillus pumilus NCIMB 10822 NAT test plates, our preliminary conclusion is that the animal samples are suspected for the presence of tetracycline, macrolide, and β-lactam antibiotics.

Introduction

Screening of food-producing animals for the presence of antimicrobial residues is one of the main pillars of the national monitoring programs executed under the Council Directive 96/23/EC (Berendsen et al., 2011). During their life, animals are exposed to different antimicrobial substances that could leave undesirable residues in their tissues and products and thus pose risk to human health.

The presence of antimicrobial residues in animal tissues and products is a matter of concern for public health (European Commission, 2002). To protect the health of consumers, maximum residue limits (MRLs) of pharmacologically active substances in foodstuffs of animal origin have been established by the Commission Regulation (EU) No. 37/2010 (European Commission, 2010). The MRLs serve as the points for the control of residues in food of animal origin in the Member States and at border inspection posts (European Commission, 2009) and have to be properly controlled in accordance with the Council Directive 96/23/EC (European Commission, 1996). The methods used for official control of residues must be validated according to common procedures and performance criteria established by the Commission Decision 2002/657/EC (European Commission, 2002).

The strategy of current residue control is based on two sequential steps: screening and confirmation. A screening method means a method that is used to detect the presence of a substance or a class of substances at the level of interest (at or below their MRLs). These methods have the capability for a high sample throughput and are used to sift large number of samples for potential noncompliant results (European Commission, 2002; Kožárová et al., 2009). Microbial inhibition tests meet the criteria set for screening methods. Principally, two main test formats can be distinguished: the tube test and plate test systems. A tube (or a vial, or an ampoule) test contains an agar medium, seeded with a single sensitive test bacterium, supplemented with a pH or redox indicator. The sample is applied to the test tube. At the appropriate temperature, the bacteria start to grow and produce acid, which will cause a colour change. The presence of antimicrobial compound becomes apparent from a delayed or absent colour change, due to impaired growth of the test bacterium. The most commonly used bacterial strain for this type of test is Bacillus stearothermophilus var. calidolactis. In (multi-)plate assays, the sample is applied on top of, or in punch holes in, an inoculated agar medium. After incubation, the presence of an antibiotic will show as a growth inhibition zone around the sample. The diameter of the inhibition zone theoretically correlates with the concentration of the antimicrobial compound (Pikkenmaat et al., 2009). The plate methods developed so far are using one to seven plates, different pH and media combinations, with one or different bacterial strains to improve the capability detection of different antibiotic groups (Dang et al., 2011).

According to this test principle based on a specific inhibitory reaction between a sensitive bacterial strain and the antimicrobial present in the sample, many microbial inhibition tests have been developed for the screening of antimicrobial residues in the tissues of slaughter animals. Among these tests, the Premi®Test developed by DSM Food Specialties (DSM Food Specialties, Delft, the Netherlands), now part of the R-Biopharm (R-Biopharm, Darmstadt, Germany) product line as of 2011 as the tube test, and the Four-Plate Test (FPT) (Bogaerts and Wolf, 1980) and the Screening Test for Antibiotic Residues (STAR) developed at the EU Community Reference Laboratory AFFSA in Fougères (France) as plate tests have been most widely used in official residue screening (Okerman and van Hoof, 1998; Gaudin et al., 2004, 2008, 2010; Stead et al., 2004, 2005; Magalhães et al., 2012). Premi®Test covers a broad spectrum of antimicrobial substances and offers a first tool (prescreening) to assay the presence of residues in animal tissues. Because the Premi®Test cannot distinguish the families of antibiotics, the samples should be further subjected to an analysis by the FPT or the STAR (screening). These tests comprise four or five test plates, respectively. Each plate is preferentially sensitive for one or two families of antimicrobials. The combination of tube and plate tests constitutes an efficient mean of controlling antimicrobial residues in animal tissues. An ideal screening method should be accurate.
and detect all licensed antimicrobials at or below their MRLs. In the recent years, we have again seen a progress in the development of microbial screening methods that show a highly improved performance with respect to certain antimicrobials. One of new tests designed for rapid detection of antibiotics in meat is a tube test Total Antibiotics. It was developed by the EuroClone S.p.A. [Euroclone S.p.A., Pero (MI), Italy]. In contrast to the existing tube tests, the Total Antibiotics provides increased sensitivity to reach the MRL and decrease the number of false positive results due to different preparation of meat samples before the analysis. The samples are extracted with an extraction buffer solution and the clear supernatant is directly used in the test. The second of the new tests introduced for the screening of antimicrobial residues in slaughter animals is the plate test, the Nouws Antibiotic Test (NAT) developed by Pikkemaat et al. (2008). This test is based on the analysis of renal pelvis fluid and comprises five test plates individually optimized, enabling group specific identification. Since the STAR and the PremiTest are officially approved for screening food-producing animals and their products for residues of antimicrobial substances in Slovakia (Slovak Regulation 2006a, 2006b), we aimed to compare these two official methods mentioned above with two new alternatives, the NAT (Pikkemaat et al., 2008) and the Total Antibiotics (EuroClone S.p.A), for the screening of antimicrobial residues in the tissues of food-producing animals.

Materials and methods

Sample material

A total of 142 samples of food-producing animals (bovine liver (20), kidney (17) and meat (7); porcine liver (23), kidney (21) and meat (9), and chicken liver (19), kidney (8) and meat (18) were collected from different slaughterhouses and retail operations in Slovakia during the 18-month period from March 2012 to September 2013. Chicken kidneys were manually removed from the back of the whole chicken carcasses used for the screening. The animal samples were stored in a freezer at -20°C until the analysis.

Screening Test for Antibiotic Residues

Media and strains

The following culture media were used: test agar pH 6.0 (Merck 10663; Merck, Darmstadt, Germany), test agar pH 8.0 (Merck 10664), antibiotic medium 11 (Difco 259310; Difco, Detroit, USA), Diagnostic Sensitive Test (DST) agar (Oxoid CM 261; Oxoid, Basingstoke, UK). The following bacterial strains were used: Bacillus subtilis BGA (Merck 10649) and Bacillus stearothermophilus var. calidolactis ATCC 10149 (Merck 1.11499) as the commercial spore suspensions, Bacillus cereus ATCC 11778 (Czech Collection of Microorganisms, Brno, Czech Republic), Kocuria rhizophila ATCC 9341 (Czech Collection of Microorganisms) and Escherichia coli ATCC 11303 (Czech Collection of Microorganisms) as freeze-dried cultures.

Preparation of test plates

Antibiotic medium 11 adjusted to pH 8.0 and DST agar adjusted to pH 7.4 were inoculated with ready-to-use commercial spore suspension Bacillus subtilis BGA and Bacillus stearothermophilus var. calidolactis ATCC 10149 at the concentrations of 5×10⁶ spores/mL and 5×10⁵ spores/mL, respectively. DST agar was further supplemented with trimetoprim (TMP) (Fluka 92131; Fluka, Buchs, Switzerland) to obtain a final concentration of 0.005 µg/mL in the agar medium. Test agar pH 6 was inoculated with bacterial suspension Bacillus cereus ATCC 11778 to give a final concentration of 3×10⁴ germs/mL, and test agar pH 8.0 was inoculated with bacterial suspension Kocuria rhizophila ATCC 9341 and Escherichia coli ATCC 11303 to give a final concentration of 5×10⁴ germs/mL and 10⁵ germs/mL, respectively. Finally, the inoculated agar media at a volume of 5 mL were poured into Petri dishes of 90 mm in diameter. The culture media, the revival of freeze-dried cultures and the bacterial suspensions were prepared according to the manufacturer’s instructions.

Preparation and screening of the samples

A cylindrical core obtained from each frozen sample (livers, kidneys and muscles from cattle, pigs and chickens) using a sterile cork borer (9 mm) was cut into slices of 2 mm in thickness with a sterile lancet. The slices were then placed opposite each other in each of five test plates. The test plates were incubated as follows: the plates seeded with Bacillus subtilis BGA and Bacillus cereus ATCC 11778 at 30°C for at least 18 h, the plates seeded with Kocuria rhizophila ATCC 9341 and Escherichia coli ATCC 11303 at 37°C for at least 24 h, and the plates seeded with Bacillus stearothermophilus var. calidolactis ATCC 10149 at 55°C for 12 to 15 h.

Reading the test results

The samples were considered positive, if they gave the inhibition zone equal or superior to 2 mm in width 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 in width on plates seeded with Bacillus stearothermophilus var. calidolactis ATCC 10149. To verify that the operating conditions were systematically respected, a quality control on 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 antibiotics prepared and stored according to the procedures set by the method. The width of the inhibition zone was measured as the distance between the edge of the slice of the tissue or the disc and the outer limit of the inhibition zone in mm using a digital calliper (Mitutoyo, Kawasaki, Japan) with a precision of 0.01 mm.

Orientation

Bacillus subtilis BGA test plates are specific for aminoglycosides, Kocuria rhizophila ATCC 9341 test plates for macrolides, Bacillus cereus ATCC 11778 test plates for tetracyclines, Escherichia coli ATCC 11303 test plates for quinolones and Bacillus stearothermophilus var. calidolactis ATCC 10149 test plates for β-lactams and sulphonamides.

Nouws Antibiotic Test

Media and strains

The following culture media were used: Iso-sensitest agar (Oxoid), Plate Count Agar (Difco), DST agar (Oxoid). The following bacterial strains were used: Bacillus subtilis BGA (Merck 10649) as a commercial spore suspension, and Bacillus cereus ATCC 11778 (Czech Collection of Microorganisms), Kocuria rhizophila ATCC 9341 (Czech Collection of Microorganisms), Yersinia ruckeri NCIMB 13282 (National Collection of Industrial, Food and Marine Bacteria /NCIMB/, Aberdeen, UK) and Bacillus pumilus NCIMB 10822 (strain name CN 607) (National Collection of Industrial, Food and Marine Bacteria) as freeze-dried cultures.

Preparation of test plates

Plate Count Agar adjusted to pH 8.0 was inoculated with ready-to-use commercial spore suspension Bacillus subtilis BGA at the concentration of 10⁶ spores/mL. Plate Count Agar adjusted to pH 6.5 was inoculated with bacterial suspension Yersinia ruckeri NCIMB 13282 to
give a final concentration of 10^6 germs/mL. Iso-
sensitest agar adjusted to pH 6.0 was inoculat-
ed with bacterial suspension Bacillus cereus
ATCC 11778 to give a final concentration of 10^6
erms/mL. Iso-sensitest agar adjusted to pH 8.0 was inoculated with bacterial suspension
Kocuria rhizophila ATCC 9341 to give a final concentration of 10^6 germs/mL. DST agar
adjusted to pH 7.0 was inoculated with bacteri-
sal suspension Bacillus pumilus NCIMB 10822
to give a final concentration of 10^6 germs/mL.
DST agar was further supplemented with TMP
to obtain a final concentration of 0.007 µg/mL
in the agar medium and chloramphenicol
(CAP) (Serva 16785; Serva, Heidelberg,
Germany) to obtain a final concentration of
0.625 µg/mL, respectively. The inoculated agar
media were poured into Petri dishes of 90 mm
diameter as a 2.5 mm thick layer, except for agar medium inoculated with Bacillus pumilus
NCIMB 10822 which was 3 mm thick. After the
agar media solidified, four holes at the diam-
ters of 14 mm were punched using a sterile
cork borer in each test plate. The culture media,
the revival of freeze-dried cultures and the
bacterial suspensions were prepared according
to the manufacturer's instructions.

Preparation and screening of the samples
The juice from frozen samples was used for
screening. The juice was prepared by thawing the
samples obtained by using a sterile cork borer in a microwave oven on defrost setting
for 2 min. Filter paper discs at the diameters of
12.7 mm (A 2668 0127; Albet-Hahnemühle,
Dassel, Germany) moistened with 100 µL
of tissue juice were placed into the punch holes
and supplemented with a plate specific syner-
gistic buffer, namely 200 µL 0.1 M phosphate
buffer pH 6.0 (Bacillus cereus ATCC 11778),
200 µL 0.1 M phosphate buffer pH 6.5 (Versinia
ruckeri NCIMB 13282), 200 µL 1.5 M phosphate
buffer pH 6.0 (Kocuria rhizophila ATCC 9341),
200 µL 0.1 M Tris buffer pH 8.5 (Bacillus sub-
tilis BGA) and 300 µL 0.133 M phosphate buffer
pH 8.0 (Bacillus pumilus NCIMB 10822). The
test plates were incubated as follows: the
plates seeded with Bacillus cereus ATCC 11778
and Versinia ruckeri NCIMB 13282 at 30°C for 18
h, and the plates seeded with Kocuria rhizo-
phila ATCC 9341, Bacillus subtilis BGA and
Bacillus pumilus NCIMB 10822 at 37°C for 18
h. The phosphate buffers were prepared from a
combination of the monobasic and dibasic
salts, titrated against each other to the correct
pH, and the Tris buffer was prepared by dis-
solving the Tris powder (Merck 108387) in
demineralised water and titration with
hydrochloric acid (HCl, Merck 1090527) until
the correct pH was reached.

Reading the test results
Samples were considered positive, if they
gave the inhibition zone around the punch
hole and the diameter of the inhibition zone
including the punch hole of 14 mm exceeded
15 mm. The width of the inhibition zones was
measured in mm using a digital calliper with
a precision of 0.1 mm. To verify that the oper-
ating conditions were systematically respect-
ed, a quality control on each test plate was per-
formed using a 12.7 mm paper disc soaked
with 100 µL of standard solutions of control
antibiotics prepared and stored according to
the procedures set by the method.

Orientation
Bacillus subtilis BGA test plates are specific
for aminoglycosides, Kocuria rhizophila ATCC
9341 test plates for macrolides and β-lactams,
Bacillus cereus ATCC 11778 test plates for
tetracyclines, Versinia ruckeri NCIMB 13282 test
plates for quinolones, and Bacillus pumilus
NCIMB 10822 test plates for sulphonamides.

Premi®Test
Product detail
A commercial antibiotic broad spectrum test
kit supplied by R-Biopharm AG (Germany) containing 25 or 100 ready-to-use ampolles with Bacillus stearothermophilus
var. calidolactis in a solid agar medium.

Preparation and screening of the samples
Premi®Test was performed according to the
manufacturer's instructions. An aliquot of 2.5
µL sample obtained by using a sterile cork borer was minced with a lancet and trans-
ferred in the 15 mL tube containing 10 mL of
working extraction buffer prepared by dilution of extraction buffer provided in the proportion of 1:10 with demineralised water. The sample
was shaken vigorously and incubated at 37°C
for 2 h. The clear supernatant was used for
analysis. A 200-µL sample was pipetted onto
agar in the test tube. The test tube was tightly
capped and placed in a digital dry bath
(Labnet) for approximately 3 h at 65°C. The
test was completed when the negative control
sample turned yellow.

Reading the test results
Sample was considered negative for antibiot-
ic, if a medium colour turned from purple to yel-
low. Sample was considered positive for antibiot-
ic, if a medium colour remained unchanged
(purple). All the possible colour shades between yellow and purple, which may give rise to doubts,
are due to a decrease in the bacterial growth rate
cased by the presence of inhibition factors in
the samples, such as antibiotics substances
just below the detection limits. These samples
were considered as dubious. All dubious samples
were tested again to confirm the results
obtained (intermediate colour).

Statistical analysis
Agreement between the tests results was
performed by calculating the kappa (K) value
at 95% confidence interval using Kappa with
linear weighting (http://vassarstats.net
/kappa.html). A K-value of 0.60 or more was
considered a good level of agreement.
### Results and discussion

The results of screening for the presence of antimicrobials residues in the liver, kidneys and muscles from cattle, pigs and chickens by four microbial inhibition tests are presented in Table 1. In the case of plate methods, Table 1 shows only positive results detected on the STAR test plates seeded with Bacillus cereus ATCC 11778, Kocuria rhizophila ATCC 9341 and Bacillus stearothermophilus var. calidolactis ATCC, and on the NAT test plates seeded with B. cereus ATCC 11778 and K. rhizophila ATCC 9341. No inhibition zones were observed on the test plates seeded with other test strains of both methods used.

Summarizing the results of the microbial screening, out of the 142 animal samples screened for antimicrobial residues by all four microbial inhibition tests, 39 samples (27.5%) yielded a positive or dubious result on one or more tests as follows: 4 samples in all four tests, 14 samples in three tests, 13 samples in two tests, and finally 8 samples in one test.

Evaluating the results of the individual microbial inhibition tests, the most positive results were detected by the STAR, followed by the Total Antibiotics, the PremiTest, and the NAT. Comparing the plate tests and the tube tests, the plate tests showed more positive results than the tube tests. Comparing the same test formats with each other, the STAR

### Table 1. Overview of the results detected by screening of animal samples for the presence of antimicrobial residues by using the STAR, NAT, PremiTest, and Total Antibiotics tests.

| Animal | Matrix | Sample number | B. cereus ATCC 11778 | K. rhizophila ATCC 341 | B. stearothermophilus var. calidolactis ATCC 10149 | B. cereus ATCC 11778 | K. rhizophila ATCC 9341 | PreminiTest | Total Antibiotics |
|--------|--------|---------------|----------------------|-----------------------|-----------------------------------------------|----------------------|-----------------------|-------------|------------------|
| Cattle | Liver (20/5^\textsuperscript{\textregistered}) | 2 | - | 2.29±0.35 | 4.86±0.97 | - | - | + | + |
|        |        | 4 | - | 3.21±1.89 | 4.54±0.77 | - | - | ± | ± |
|        |        | 5 | - | 3.08±0.78 | 4.65±0.84 | - | - | + | + |
|        |        | 9 | - | 3.44±0.79 | 5.22±1.87 | - | - | - | - |
|        |        | 20 | - | 3.52±1.01 | - | - | - | - | ± |
| Kidney (17/5^\textsuperscript{\textregistered}) | 1 | - | 3.03±0.97 | 5.57±1.06 | - | 23.41±0.92 | ± | ± |
|        |        | 7 | - | 6.45±0.34 | 7.20±0.84 | - | 22.67±0.10 | + | + |
|        |        | 8 | - | 2.76±0.75 | 5.58±0.67 | - | 18.79±0.65 | - | - |
|        |        | 11 | - | 2.24±0.63 | 5.73±1.23 | - | 17.25±0.33 | - | - |
|        |        | 14 | - | - | 3.79±0.55 | - | - | - | - |
| Muscle (7/3^\textsuperscript{\textregistered}) | 3 | - | 3.17±0.53 | 3.65±0.68 | - | - | - | - |
|        |        | 4 | - | 3.34±0.61 | 3.18±0.94 | - | - | ± | ± |
|        |        | 5 | 3.58±0.68 | 2.11±0.35 | - | - | - | - |
| Pigs   | Liver (23/5^\textsuperscript{\textregistered}) | 3 | - | - | 7.84±1.06 | - | - | - | - |
|        |        | 7 | - | - | 5.47±0.94 | - | - | - | + |
|        |        | 8 | - | 2.88±0.50 | 5.36±0.88 | - | - | - | ± |
|        |        | 12 | - | 3.30±0.67 | 8.34±1.05 | - | - | - | - |
|        |        | 19 | - | - | 3.66±0.56 | - | - | - | - |
| Kidney (21/5^\textsuperscript{\textregistered}) | 2 | - | 3.55±0.87 | 7.65±1.21 | - | 16.58±1.03 | ± | ± |
|        |        | 5 | - | - | 7.34±0.69 | - | - | - | - |
|        |        | 9 | - | - | 7.91±1.73 | - | - | + | + |
|        |        | 11 | - | - | 6.32±1.06 | - | - | + | + |
|        |        | 17 | 9.65±1.89 | 5.69±0.86 | 23.21±0.72 | - | + | + | ± |
| Muscle (9/2^\textsuperscript{\textregistered}) | 1 | - | 1.76±0.51 | 5.30±0.95 | - | - | - | + |
|        |        | 6 | 1.89±0.38 | 4.27±0.88 | - | - | - | - |
| Chickens | Liver (19/5^\textsuperscript{\textregistered}) | 3 | - | 2.86±0.88 | 5.91±1.71 | - | - | + | + |
|        |        | 8 | 9.17±1.36 | - | 6.72±1.33 | - | - | + | + |
|        |        | 14 | - | - | 9.97±0.85 | - | - | + | + |
|        |        | 15 | - | - | 7.72±1.26 | - | - | + | + |
|        |        | 17 | - | - | 9.53±1.51 | - | - | + | + |
| Kidney (8/5^\textsuperscript{\textregistered}) | 1 | 9.46±0.93 | 5.22±0.98 | - | - | + | + | ± | ± |
|        |        | 4 | - | - | 3.72±0.74 | - | - | - | - |
|        |        | 5 | - | - | 4.02±0.28 | - | - | - | ± |
|        |        | 6 | - | - | 9.88±1.76 | - | - | + | + |
|        |        | 8 | - | - | 8.72±1.74 | - | - | + | + |
| Muscle (18/4^\textsuperscript{\textregistered}) | 6 | - | - | 4.48±1.14 | - | - | + | + |
|        |        | 10 | 4.52±0.79 | 2.70±0.39 | - | - | - | - |
|        |        | 16 | - | - | 4.83±0.22 | - | - | + | + |
|        |        | 18 | - | - | 3.06±1.11 | - | - | + | ± |

STAR: Screening Test for Antibiotic Residues; NAT: Noosev Antibiotic Test. *K-value=0.65; **K-value=0.44; ***K-value=0.32; ♦positive results of the samples concerned; ♦percentage of the positive results of the samples concerned; †positive results, ‡negative results; ± dubious results. Data concerning sizes of inhibition zones are presented as mean±standard deviation of six measures.
showed a much higher number of positive results than the NAT, and the Total Antibiotics showed a higher number of positive or dubious results than the Premi® Test. STAR yielded 39 positive results on three test plates. Five samples showed inhibition on the test plates seeded with B. cereus ATCC 11778 and B. steatorrhomophilus var. calidolactis ATCC, 16 samples showed inhibition on the test plates seeded with K. rhizophila ATCC 9341 and B. steatorrhomophilus var. calidolactis ATCC 10149, and 18 samples showed inhibition only on the test plates seeded with Bacillus steatorrhomophilus var. calidolactis ATCC 10149. The mean diameters of the inhibition zones on the Bacillus cereus ATCC 11778 test plates ranged from 3.58±0.68 to 9.65±1.89 mm, the mean diameters of the inhibition zones on the Kocuria rhizophila ATCC 9341 test plates ranged from 1.76±0.51 to 6.45±0.34 mm, and the mean diameters of the inhibition zones on the B. steatorrhomophilus var. calidolactis ATCC 10149 test plates ranged from 3.18±0.94 to 9.98±1.76 mm. NAT yielded a total of 6 positive results on two test plates. One sample showed inhibition on the test plates seeded with B. cereus ATCC 11778, and 5 samples showed inhibition on the test plates seeded with K. rhizophila ATCC 9341. The mean diameter of the inhibition zones on the B. cereus ATCC 11778 test plates was 23.21±0.72 and the mean diameters of the inhibition zones on the K. rhizophila ATCC 9341 test plates ranged from 16.98±1.03 to 23.41±0.92 mm. Premi® Test yielded a total of 16 positive results (3 of them dubious). Total Antibiotics yielded a total of 31 positive results (9 of them dubious). The inhibited test strain of both tube tests was B. steatorrhomophilus var. calidolac-

tis.

Statistical agreement between the results of the individual microbial inhibition tests in the same test formats was estimated using the K-value. In spite of the fact that more positive results, based on the absence of a clear colour change from purple to yellow, were visually detected by the Total antibiotics than by the Premi® Test; the K-value observed between the Premi® Test and the Total antibiotics was 0.625 (the 0.95% lower and upper confidence limits: 0.45-0.80), which indicates that these tube tests do not differ significantly. However, when the STAR and the NAT were compared with the linear weighted Kappa test, we found that both plate tests differ significantly when B. cereus ATCC 11778 (K-value 0.32, 0.95% lower and upper confidence limits: 0.0-0.72) or K. rhizophila ATCC 9341 (K-value 0.44, 0.95% lower and upper confidence limits: 0.13-0.76) were used to detect antibiotic residues.

According to the sensitivity of the test strains of the multi-plate methods, the inhibition zones appeared on the B. cereus ATCC 11778, K. rhizophila ATCC 9341 and B. stearothermophilus var. calidolactis STAR test plates indicated the presence of tetracyclines, macrolides, and ß-lactams and sulphonamides, respectively. The inhibition zones appeared on the Bacillus cereus ATCC 11778 and K. rhizophila ATCC 9341 NAT test plates indicated the presence of tetracyclines, and macrolides and ß-lactams, respectively. According to the sensitivity of the test strain of both tube tests, B. stearothermophilus var. calidolactis is very sensitive to many antibiotics, especially ß-lac-tam antibiotics and sulphonamides. To deter-
mind more specific the antibiotic after positive results, the samples showed the positive results on the B. stearothermophilus var. calidolactis STAR test plates and the K. rhizophila ATCC 9341 NAT test plates were further retest-
ed by both tube tests with the use of penicil-
nase (Sigma P0389; Sigma-Aldrich, St Louis, MO, USA) to confirm the presence of ß-lactam antibiotics. The post screening was performed according to the Premi® Test and the Total Antibiotics manufacturer’s instructions. One bovine kidney sample was positive for the presence of ß-lactam antibiotics by both tube tests.

Evaluating the results of the food animals, chickens showed the most positive results, fol-

lowed by cattle and pigs. Of the 45 chicken samples screened for antimicrobial residues, 14 samples (31.1%) consisting of 5 livers, 5 kidneys and 4 muscles yielded a positive result. Out of the 44 bovine samples screened for antimicrobial residues, 13 samples (29.5%) consisting of 5 livers, 5 kidneys and 3 muscles yielded a positive result. Of the 53 porcine samples screened for antimicrobial residues, 12 samples (22.6%) consisting of 5 livers, 5 kidneys and 2 muscles yielded a positive result.

The studies comparing multi-plate and tube microbiological inhibition tests for the screen-
ing of the presence of antimicrobial residues in slaughter animals or routine monitoring samples usually showed a higher number of positive samples by the multi-plate tests. Pikkemaat et al. (2009) detected a much higher number of suspect samples by two multi-

plate test systems like the STAR and the NAT, compared to the tube test system like the Premi® Test. Of the 591 slaughter animals examined, the STAR showed positive results for 34 animals on one or more test plates, the NAT showed positive results for 40 animals on only one of the five test plates, and the Premi® Test showed positive results for 6 ani-
mals. The authors further analysed all the NAT positive samples by the NAT-meat and NAT-kid-

necy post-screening tests. They found out that 14 samples remained positive by the NAT-meat and 17 samples remained positive by the NAT-kidney. The number of the STAR and the NAT positive results were comparable. The authors concluded that although multi-plate tests are more laborious and require a longer incubation time, they use more than one indicator organism to target the broad range of veteri-
nary antibiotics and track down many more residue containing samples. They also have the capability to narrow down the antibiotic family identity of a residue. The STAR test plates indicated the presence of all groups of antibiotics, mainly tetracyclines followed by ß-
lactams and sulphonamides, macrolides, aminoglycosides and quinolines, and the NAT test plates also indicated mainly tetracyclines followed by aminoglycosides, ß-lactams and macrolides, and sulphonamides. To determine more specific the antibiotic after the Premi® Test positive results, the authors fur-

ther analysed all the positive samples with a penicillinase post-screening test. They detect-
ed that two positive samples were clearly beta-

lactams, since the addition of penicillinase counteracted the inhibition.

Pikkemaat et al. (2011) in their study evalu-

ated and compared the performance of the FPT and the NAT as the multi-plate test and the Premi® Test as the tube test by parallel analysis of 735 slaughter animals. They detected that the FPT yielded a very low number of positive samples with the significant inhibition zones observed with only two samples on two test plates, the NAT yielded a total of 36 positive results with the initial screening of renal pelvis fluid on three test plates, and the Premi® Test-muscle and Premi® Test-kidney showed 9 muscle samples and 30 kidney sam-

ples positive, respectively. While the number of kidney positive results was similar to the num-

ber of suspect samples obtained after the NAT screening, the overlap of samples suspect in both tests was limited to eight samples. The FPT test plates indicated the presence of tetracycline and macrolide antibiotics. The NAT test plates indicated the presence of tetracyclines followed by aminoglycosides and one sulphonamide. The Premi® Test after retesting the positive samples with penicillinase confirmed none of the muscle and kidney samples positive for the presence of ß-lactam antibi-

otics.

Currently, there are no further published studies dealing with the comparison of the NAT test with the other multi-plate tests, and no studies dealing with the comparison of the
Total Antibiotics with the other tube tests. Considering the results obtained in the studies mentioned above and because the STAR and the Premi® Test are the only multi-plate and the tube agar diffusion methods officially approved for screening food-producing animals and their products for residues of veterinary drugs in Slovakia today, we focused in this study on the comparison of the results of two officially approved methods with two new recently introduced tests based on same test principle and the same test formats for the screening of antimicrobial residues in the tissues of food-producing animals.

Summarizing our results of the multi-plate screening tests we can assume that by the STAR the samples were positive for the presence of tetracycline, macrolide, and β-lactam antibiotics and sulphonamides, and by the NAT the samples were positive for the presence of tetracyclines, and macrolide and β-lactam antibiotics. Both STAR and NAT showed the inhibition of the same test strains of both methods in one sample on the B. cereus ATCC 11778 test plates and in five samples on the K. rhizophila ATCC 9341 test plates. Since B. cereus ATCC 11778 of both methods is specific for tetracyclines, and macrolide and β-lactam antibiotics. Both STAR and NAT showed the inhibition of the same test strains of both methods in one sample on the B. cereus ATCC 11778 test plates and in five samples on the K. rhizophila ATCC 9341 test plates. Since B. cereus ATCC 11778 of both methods is specific for tetracyclines, and macrolide and β-lactam antibiotics, respectively. Because of the potential for the presence of sulphonamide residues in the positive samples detected on the B. stearothermophilus var. calidolactis ATCC 10149 STAR test plates and no positive samples detected on the B. pumilus NCIMB 10822 NAT test plates specific for sulphonamides, we assume these five positive samples suspected for the presence of macrolide and β-lactam antibiotics, respectively. Because of the potential for the presence of sulphonamide residues in the positive samples detected on the B. stearothermophilus var. calidolactis ATCC 10149 STAR test plates and no positive samples detected on the B. pumilus NCIMB 10822 NAT test plates specific for sulphonamides, we assume these five positive samples suspected for the presence of macrolide and β-lactam antibiotics.

By comparing the results obtained in our study and the results published by Pikkemaat et al. (2009, 2011), we expected to have more comparable results. Although in our study the NAT yielded a lower number of positive results than the STAR, possibly due to differences in the sample preparation and the screening of tissue fluid obtained from frozen samples, the NAT must not be underevaluated. Our other partial results (Kožárová and Gondová, 2012) obtained by the screening of food matrices fortified with sulphamethazine (SMZ) standard showed more comparable results obtained by both methods used. The detection capacities of B. stearothermophilus var. calidolactis ATCC 10149 of the STAR and B. pumilus NCIMB 10822 of the NAT for SMZ were 200 µg/L and 100 µg/L, respectively. When the fortified food samples showing positive responses are sub- ject to further investigation by a specific confirmatory method that provides full or complementary information enabling the substance to be unequivocally identified and, if necessary, quantified at the level of interest (European Commission, 2002).

All the microbial inhibition tests used in our study showed the ability to characterize the samples as positive. The higher numbers of positive results were detected by the officially approved multi-plate test, the STAR, and the unofficial, recently introduced tube test, the Total Antibiotics. The higher number of positives detected by both microbial inhibition tests allowed us to come to the preliminary conclusion that these tests showed a higher level of sensitivity to the antibiotics presented in the screened samples than the NAT and the Premi® Test. However, more detailed studies are needed to reach a final conclusion on whether two recently introduced screening tests, the NAT and the Total Antibiotics, are more suitable alternatives for the commercial screening purposes. Microbial inhibition tests still form the first line of defence in monitoring the presence of antibiotic residues in food of animal origin intended for human consumption, it is therefore crucial to have accurate screening tests at hand.

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