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<table>
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Screening methods for detection of antibiotic residues in slaughter animals: comparison of the EU-four plate method, the Nouws Antibiotic Test and the Premi® Test (applied to muscle and kidney)

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Abstract

Microbial growth inhibition tests are widely used as the primary screening approach for the detection of antibiotic residues in slaughter animals. In this study we evaluated and compared the performance of the EU-four plate method (EU4pt), the Nouws Antibiotic Test (NAT), and a commercial ampoule test, the Premi® Test (applied to both muscle and kidney), by parallel analysis of 735 slaughter animals. The EU4pt only showed significant inhibition with two muscle samples containing 305 µg kg\(^{-1}\) doxycycline and 648 µg kg\(^{-1}\) tulathromycin, while an MRL violation of 1100 µg kg\(^{-1}\) sulfamethazine remained unnoticed. Premi® Test-muscle only detected the sulfamethazine containing sample, all other (1.1%) suspect samples appeared false-positive results. The same test applied to kidney yielded 4.1 % suspect samples, while the NAT-screening (based on analysis of renal pelvis fluid) showed 4.9 % suspect results. The vast majority of these samples contained tetracycline and/or aminoglycoside residues. Premi® Test-kidney appeared more sensitive to aminoglycosides than the NAT-screening, which failed to detect an MRL violation of 870 µg kg\(^{-1}\) gentamicin in kidney. Detection of <MRL levels of tetracycline residues by the NAT proved its suitability for this residue group. Whether Premi® Test is sufficiently sensitive for accurate tetracycline detection in kidney remained doubtful, although changing over to kidney definitely improved the suitability of Premi® Test for detection of residues in slaughter animals.

Keywords: antibiotic residues; microbial screening method; Premi® Test; Nouws Antibiotic Test; EU-four plate test
Introduction

Worldwide, a significant percentage of all antibiotic drugs is administered to farm animals, either for curative, prophylactic or growth promotion purposes. In order to reduce the risk of harmful (levels of) residues entering the food chain, veterinary drugs are subjected to stringent assessment and legislation concerning safety evaluation, registration, determining maximum residue limits (MRLs) and withdrawal periods. To enforce these administrative measures, most developed countries have extensive monitoring and surveillance programs in place.

Within the European Union each member state is obliged to carry out a national residue monitoring plan. Directive 96/23/EC (European Commission 1996) establishes the frequencies and level of sampling and the groups of substances to be controlled for each food commodity. These extensive statutory screening programs require inexpensive high-throughput methods. Different from, for example, the US, in the EU there are no mandatory or reference methods. Each national authority is free to implement the method it considers most suitable, though methods should be validated according to specific guidelines and the method characteristics have to meet pre-defined criteria (European Commission 2002; Anonymous 2010).

Large scale screening of animal products for the presence of residues of antibiotics is commonly applied using microbial screening methods. These methods are based on bacterial growth inhibition of a sensitive test-bacterium, and essentially originate from procedures testing pharmaceutical preparations and body fluids (Grove and Randall 1955). In contrast to the field of the instrumental confirmatory methods, where major efforts are spend on method development, establishing EU Maximum Residue Limits has induced only minor efforts on method enhancement and implementation of microbial screening methods. It has been shown that the current state-of-the-art is often not sufficient to support legislative requirements (Pikkemaat 2009; Pikkemaat et al. 2009a). Since these methods form the foundation of the residue monitoring system, this situation leads to inadequate consumer protection. In order to draw more attention to this problem, we
evaluated the performance of two commonly used screening methods and compared them with the method routinely applied in the Netherlands, the Nouws Antibiotic Test (NAT) (Pikkemaat et al. 2008; Pikkemaat et al. 2009b).

First of all the EU four-plate method (EU4pt) was assessed. This method was first published in 1980 (Bogaerts and Wolf 1980) and is based on three Bacillus subtilis based test plates and a fourth Micrococcus luteus plate (ATCC 9341, renamed to Kocuria rhizophila (Tang and Gillevet 2003). Within the EU this method served as a reference until it was decided to determine acceptable residue limits on a more scientifically based approach (the effectuation of Council Regulation (EEC) 2377/90 (European Commission 1990)) and MRLs were set at levels beyond the detection capability of this method.

Although the EU4pt is generally recognized not to be sufficiently sensitive, it is still used in many laboratories (Berendsen et al. 2010; Gaudin et al. 2008).

As an example of an ampoule or tube test, Premi®Test (DSM) was included. This type of test is based on Bacillus stearothermophilus, a bacterium particularly sensitive to beta-lactam antibiotics. Recently the Premi®Test and a similar test, the Explorer (Zeu-Inmunotec), have gone through an extensive validation carried out by the EU Community reference laboratory (Gaudin et al. 2008, Gaudin et al. 2009). In particular with respect to tetracyclines, it has been shown that Premi®Test lacks sufficient sensitivity to detect these antibiotics at the MRL in muscle (Pikkemaat et al. 2009a; Okerman et al. 2004).

According to the manufacturer DSM, Premi®Test is also suitable for other matrices, including kidney. Since for tetracyclines (and several other antibiotics) the MRL in kidney tissue is much higher (tetracyclines: 100 µg kg⁻¹ in muscle, vs. 600 µg kg⁻¹ in kidney), this organ could be a more suitable matrix for antibiotic screening. To investigate the potential of applying kidney instead of muscle, both matrices were tested in parallel.

The Nouws Antibiotic Test (NAT) comprises a 5-plate (residue group-specific) initial screening based on the analysis of renal pelvis-fluid (Pikkemaat et al. 2008), and two subsequent post-screening tests for further analysis of kidney and/or muscle of suspect...
animals (Pikkemaat et al. 2009a; Pikkemaat et al. 2009b). The EU4pt, the NAT and the
Premi® Test (muscle and kidney), were performed in parallel on slaughter animals tested
within the framework of the national monitoring program.

Materials and Methods

Sample material

All animal samples analyzed in this study were taken as part of the national monitoring
program. After slaughter, one of the kidneys and a piece of lean muscle were transported
cool to the laboratory and analyzed the next day. During the months of June and October
2009, a total of 491 pigs, 156 calves, 75 cows, 9 sheep, two goats and two horses (a total
of 735 animals) were subjected to the three parallel tests.

Methods

Nouws Antibiotic Test

The Nouws Antibiotic Test is a test system involving an initial screening of renal pelvis
fluid (pre-urine) and post-screening of muscle and kidney. Initial and post-screening tests
each comprise a series of test plates, each optimized for the detection of one or two
antibiotic groups in a specific matrix. The initial screening comprises five plates: a
Bacillus cereus ATCC 1178 plate specific for tetracyclines (T), a Kocuria rhizophila
ATCC 9341 plate specific for beta-lactam antibiotics and macrolides (B&M), a Yersinia
ruckeri NCIM 13282 plate specific for quinolones (Q), a Bacillus pumilus CN 607 plate
specific for sulfonamides & diaminopyrimidines (S) and a Bacillus subtilis BGA plate
specific for aminoglycosides (A). The exact composition of the individual test plates and
the procedure were described in detail in (Pikkemaat et al. 2008). In brief, an incision is
made in the kidney and renal pelvis is collected by absorption to paper disks, which are
placed on the interface of the medulla and the cortex. Each kidney is sampled with five
paper disks, one for each test-plate. The paper disks are applied to punch holes in the test
plate and supplemented with a plate specific buffer. After overnight incubation the
emergence of a growth inhibition zone indicates the presence of antimicrobial residues in
the animal.
Suspect samples, showing an inhibition zone on one or more test plates, are additionally analyzed by post-screening of kidney and/or muscle, limited to the residue group for which the initial screening tested positive. Samples for post-screening are prepared by homogenizing kidney or muscle and isolating tissue fluid from the homogenate by centrifugation after a brief heating step. The post-screening is based on a multi-plate principle similar to the initial screening and described in detail in Pikkemaat et al. (2009b) for kidney and in Pikkemaat et al. (2009a) for muscle.

**EU four-plate test**

The EU4pt was performed essentially similar to Bogaerts and Wolf (1980). Test agar pH 6 (Merck), Antibiotic medium II (Difco) (adjusted to pH 7.2, supplemented with trimethoprim to a final concentration of 50 µg l⁻¹), and Test agar pH 8 (Merck), were inoculated with approximately 10⁴ CFU/ml *B. subtilis* BGA spores (Merck) and Test agar pH 8 (Merck) was inoculated with 10⁴ CFU ml⁻¹ *K. rhizophila* ATCC 9341. A volume of 105 ml of the inoculated growth media was poured in 245 x 245 mm square petri dishes, resulting in a layer of 2 mm thickness. Samples were prepared by freezing a thin slice of muscle briefly at -80°C and subsequently take out disks, using a cork borer with a diameter of 4 mm. Meat disks were placed on each of the four test plates, with a maximum of 24 samples per plate. As a quality control on each plate a paper disk impregnated with either 10 IE penicillin, 5 µg sulfamethazine, 0.5 µg dihydrostreptomycin or 0.5 µg tylosin was added (by adding 100 µl of a fresh 10x stock). Test plates were incubated for 14-16 hr at 30°C (*B. subtilis*) or 37°C (*K. rhizophila*).

**Premi®Test**

The Premi®Test was essentially performed according to the manufacturers instructions. The test is based on the analysis of 100 µl of liquid sample extracted from the tissue. For the preparation of kidney juice, a 10 ml centrifuge tube was filled with roughly cut pieces of kidney taken from the cortex-medulla interface. This sample was heated for 10 min. at 80°C, then cooled down and centrifuged for 10 min at 27000 x g. Sample preparation for muscle was performed in a similar way. Samples of 100 µl of supernatant were applied on the test vials and removed after 20-30 minutes of pre-incubation at room temperature.
The vials were sealed and transferred to a 64°C water bath and incubated until the majority of the samples had turned yellow, which was usually after approximately 3 hrs with muscle fluid and 4 hrs when kidney was analyzed. Samples showing a positive result, which was defined as absence of any color change, were retested the next day, including a penicillinase test for identification of beta lactam antibiotics. Only samples also positive in this second test were forwarded for chemical confirmation.

**Chemical confirmation**

Samples showing a positive (suspect) result in one of the tests, but for which the group identity could not be determined from the microbial screening result, were first analyzed using a multi-compound screening method based on high-resolution liquid chromatography combined with time-of-flight mass spectrometry (HRLC-ToF-MS) (Peters et al. 2009). In short, samples were extracted intensive for 30 min using a mixture of acetonitrile and water (6/4; v/v), after which the samples were centrifuged (15 min; 3600 g; 10°C). An aliquot of the supernatant was diluted 20 times using water and applied to a StrataX SPE column (60 mg). After elution using a methanol/acetonitrile mixture (1/1; v/v), the eluate was evaporated under a stream of nitrogen at 40°C till near dryness and the residue was re-dissolved in acetonitrile. After addition of 0.1% formic acid the extract was analyzed by HRLC-ToF-MS in the full scan mode. Chromatographic separation was achieved using a Waters Acquity UPLC system equipped with a reversed phase Waters Acquity UPLC BEH C_{18} analytical column (100 x 2.1 mm; 1.7µm) using a 0.1% formic acid/acetonitrile gradient. The mass spectrometer was operated in the electrospray positive mode using a mass range of 100 – 1000 Da.

Since aminoglycosides were not included in the scope of the multi-residue method, kidney samples were additionally tested for this antibiotic group using a quantitative analysis method using LC-MS/MS (van Holthoon et al. 2009). Quantitative analysis for other residue groups was performed with previously described LC-MS/MS methods (van Rhijn et al. 2002; Berendsen and van Rhijn 2006; Pikkemaat et al. 2009a). In short, the methods for aminoglycosides, macrolides, sulfonamides or tetracyclines methods were all...
Results and discussion

General

The Nouws Antibiotic Test (NAT) is the routine antibiotic screening method applied to slaughter animals in the Netherlands. During the months of June and October 2009 all animals which were analyzed within the framework of the national monitoring program, were tested additionally with the EU4pt and the Premi® Test, the latter applied both on muscle and kidney. In total 735 animals (pigs, bovines, sheep, goats and horses) were screened. It was anticipated that the number of treated animals would be higher during October, because deteriorating weather conditions potentially would cause increased health problems, but this was not reflected in the numbers of suspect animals. The percentage was even slightly higher in June: out of 405 animals 5.2% and 4.2% yielded a suspect result with the NAT and Premi® Test-kidney respectively, while in October 4.5% resp. 3.9% suspect results were found among the remaining 330 animals.

Out of the 735 animals, only four showed an MRL violation, all of which were pigs. Two pigs showed muscle concentrations of respectively 305 µg kg\(^{-1}\) doxycycline and 1100 µg kg\(^{-1}\) sulfamethazine. The other MRL violations were found in porcine kidneys, containing 870 µg kg\(^{-1}\) gentamicin and 5207 µg kg\(^{-1}\) neomycin. Also an animal containing a significant level (648 µg kg\(^{-1}\)) of tulathromycin in muscle was identified, but for this compound no MRL has been established in muscle, so statutorily it could not be considered an MRL violation. In some countries however a zero-tolerance policy is applied, though this remains disputable because it concerns a registered drug with a set acceptable daily intake (ADI). An overview of the results of the individual tests on these samples is shown in Table 1.

Compared to a previous study comprising 591 animals, in which we compared the performance of the NAT, the Screening Test for Antibiotic Residues (STAR) and
Premi®-Test applied to muscle, the percentage of MRL violations is somewhat lower in the current survey (0.54 vs. 0.67%), while in the previous study we did not yet have the possibility for confirmation of the aminoglycosides, which may even have led to an underestimation of the number of MRL violations.

[Insert Table 1 about here]

The EU-four plate test

Evaluating the results of the individual tests, it can be concluded that the EU4pt yielded a very low number of suspect samples. This test is based on the analysis of muscle discs, and subsequently is not suited for detecting MRL violations in kidney. Significant inhibition zones were observed with only two samples. The presence of 305 µg kg\(^{-1}\) doxycycline became apparent from all three \(B.\ subtilis\) plates, with the largest inhibition zone (23 mm) on the pH 6 plate. The tulathromycin sample (648 µg kg\(^{-1}\)) caused an inhibition zone of 23 mm on the \(K.\ rhizophila\) plate. The sulfamethazine containing sample was not detected, even though the concentration in the sample was > 10 times the MRL.

Occasionally it appeared difficult to judge the result of this test, since it easily suffered from microbial contamination causing growth on the surface of the plate around the meat disk. This sometimes was attended by growth inhibition in the agar. Some of these questionable results were additionally analyzed by the multi compound ToF-MS confirmation method but always turned out to be negative.

The Nouws Antibiotic Test

The NAT yielded a total of 36 suspect results (4.9%) with the initial screening of renal pelvis fluid. The test comprises 5 individual test plates, each one preferably sensitive to one or two groups of antibiotic residues. Of these 36 samples, one sample was specifically detected on the sulfonamide test plate (originating from the animal containing 1100 µg kg\(^{-1}\) sulfamethazine in its muscle), 24 were suspect on the tetracycline specific

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plate and another 11 on the aminoglycoside test plate. The tulathromycin containing sample showed some inhibition on the aminoglycoside test plate, but was primarily detected (showing a larger inhibition zone) on the macrolide & beta-lactam test plate.

Compared to the previous study, the percentage of tetracycline suspect animals decreased slightly (4.9 to 3.3 %) while the percentage of aminoglycoside suspect samples was nearly identical (1.5 vs. 1.4%).

Samples suspect for tetracyclines in the initial screening were subjected to post-screening of the kidney, which in routine analysis is followed by chemical confirmation in muscle (figure 1). It has been shown before that the kidney post-screening result is a more reliable indicator for the concentration in muscle than the initial (renal pelvis fluid based) screening result (Pikkemaat et al. 2009a). This is presumably caused by variations in the renal pelvis fluid available for absorption to the paper disk. Nine (37.5%) of these initially suspect samples were found negative in post screening kidney, while another seven (29%) showed an inhibition zone smaller than the control sample of 600 µg kg\(^{-1}\) oxytetracycline (OTC), which in the routine procedure is the cut-off for further chemical confirmation. So in practice only muscle samples of the remaining eight animals would be further analyzed by LC-MS/MS, though for the purpose of this study and to verify the assumption that this procedure does not yield false-compliant results, all animals showing a suspect result in the screening were fully analyzed.

[Insert Figure 1 about here]

With regard to samples showing no inhibition in the post-screening kidney, concentrations in the muscle were found to be always less than 26 µg kg\(^{-1}\) (doxycycline), while three of them were even below the limit of detection of the method (Table 2). For those that showed inhibition, but below the cut-off for chemical confirmation, concentrations were usually between 20 and 30 µg kg\(^{-1}\), with a maximum found at 42 µg kg\(^{-1}\) doxycycline. Tetracycline concentrations in samples which according to the routine procedure would be forwarded for chemical confirmation, started from minimum levels
of 25 µg kg\(^{-1}\) doxycycline and 41 µg kg\(^{-1}\) OTC. Only one out of the eight samples appeared to be a true non-compliant result, containing 305 µg kg\(^{-1}\) doxycycline.

From these results we can conclude that the procedure efficiently downsizes the number of samples requiring chemical confirmation to approximately one-third of the original suspect samples, without resulting in false-compliant results. It should be noted that the majority of the tetracycline suspect samples contained doxycycline, either alone or in combination with OTC, only in four samples OTC was the sole tetracycline residue present. This could of course be attributed to a bias caused by the method, which is more sensitive to doxycycline, however taking into account the monitoring results obtained since the introduction of the NAT in 2004, a clear overall shift from OTC to doxycycline residues is observed (M. Rapallini, unpublished results).

The other major residue group observed by the NAT-screening, were the aminoglycosides. Nine out of the 10 samples that were suspect for this group based on the renal pelvis screening, also showed an inhibition zone in the kidney post-screening. The one sample negative sample also appeared negative after LC-MS/MS analysis and should be considered as a false-positive result. Aminoglycoside residue levels in kidney ranged from 106-5027 µg kg\(^{-1}\) neomycin (the highest concentration being an MRL violation), 324-614 µg kg\(^{-1}\) dihydrostreptomycin (MRL in kidney: 1000 µg kg\(^{-1}\)) and 593 µg kg\(^{-1}\) gentamicin (Table 2). The latter yielded an NAT-screening inhibition zone of 26 mm, which is quite remarkable, since the second aminoglycoside MRL violation, 870 µg kg\(^{-1}\) gentamicin, was not noticed by the NAT-screening. This can be explained from the poor correlation between screening and final concentration in kidney and could only be prevented by direct post-screening analysis of the kidney, for which the correlation is much better. This is illustrated in Figure 2, showing screening and post-screening results of aminoglycoside positive samples.
In practice so far no cut-off (analogous to the tetracycline procedure) has been
determined, beyond which a sample should be forwarded for chemical quantification. The
current kidney post-screening results suggest a cut-off inhibition zone around 30 mm,
which is somewhat larger than the original validation study suggested (Pikkemaat et al.
2009b), but more data should be collected to enable a reliable estimation.

Additional analysis of the muscle of the suspect animals showed that aminoglycoside
residue levels in muscle were negligible. Only a single animal with a neomycin level of
3973 µg kg\(^{-1}\) in kidney also showed traces of this residue in muscle (<5 µg kg\(^{-1}\)). The
aminoglycoside residue group therefore is a very pronounced example of a non-compliant
result in kidney not necessarily reflecting a non-compliant result in muscle. This
observation implies a serious discrepancy between the outcome of control/surveillance
strategies based on the analysis of either kidney or muscle.

Premi\(^{®}\) Test-muscle

Premi\(^{®}\) Test applied to non-contaminated tissue should result in a color change from
purple to yellow, and the absence or delay in color change indicates the presence of a
growth inhibiting compound. In this study, only samples that showed a complete absence
of color change were considered suspect. The number of samples that would need further
testing in case all samples showing intermediate results were also considered,
occasionally was 5 to10-fold more, and was considered not suitable in large scale
screening. Most of these intermediate results probably should be attributed to matrix
variability. Suspect samples were always retested in presence and absence of
penicillinase for confirming the presence of beta-lactam antibiotics.

Only 9 muscle samples tested positive with the Premi\(^{®}\) Test, and none of these suspect
results were attributable to the presence of beta-lactam antibiotics. One of the positive
results concerned the sample containing 1100 µg kg\(^{-1}\) sulfamethazine, but in all other
samples the presence of antibiotic residues could not be confirmed, resulting in a very
high false-positive rate of the suspect samples. Among these false-positives was one
bovine (non-calve) sample, all others were porcine samples. The doxycycline (305 µg kg$^{-1}$) MRL violation was not detected, once more establishing that Premi® Test applied to muscle is not suitable for effective tetracycline detection. Remarkably also the sample containing 648 µg kg$^{-1}$ tulathromycin remained undetected, while in our previous study a sample containing 156 µg kg$^{-1}$ tulathromycin was found suspect (Pikkemaat et al. 2009a).

**Premi® Test-kidney**

The number of kidney samples yielding a positive Premi® Test result was substantially higher. One of the main problems generally associated with using kidney as a matrix, is the occurrence of false-positive results, probably caused by the action of enzymes present in this matrix, generally referred to as “natural growth inhibiting compounds”. A common way to circumvent this (an approach generally applied in milk analysis) is to inactivate these natural growth inhibiting compounds by heating the sample. Using Premi® Test for analysis of kidney, the manufacturer advises the incorporation of a heat-pretreatment step (10 min at 80°C) after applying the kidney juice to the ampoule.

However, in our hands this approach appeared to cause problems, since the heating/inactivation step caused a strong coloring of the growth medium. This made the ultimate color change hard to assess, and also considerably increased the required incubation time. This problem could however easily be circumvented by performing the heating step before applying the sample to the tube.

Similar to muscle analysis samples were considered positive only when there was complete absence of color change. Suspect results were first verified with a second analysis including penicillinase, however non of the kidneys appeared suspect for beta-lactam antibiotics. Applying the Premi® Test to kidney yielded 30 suspect animals. While this number is similar to the number of suspect samples obtained after the NAT screening (36), the overlap of samples suspect in both tests was limited to 8 animals. These included the sulfamethazine and doxycycline MRL violations, and a tetracycline suspect animal which was confirmed to contain 34 µg kg$^{-1}$ doxycycline in the muscle (the concentration in kidney was not determined). Furthermore the tulathromycin containing sample, and several aminoglycoside suspect samples were identified by both methods.
In twelve suspect samples the presence of antibiotic residues could not be confirmed and these should therefore be considered false-positive results (40%), notably all of these were pig samples. One sample contained 15 µg kg⁻¹ tylosin, but considering the reported sensitivity for muscle, which is 50-100 µg kg⁻¹, it seems somewhat unlikely that this low concentration actually caused the inhibition. All other suspect samples appeared to contain aminoglycoside residues (Table 2). Most of them contained neomycin, with concentrations starting from around 470 µg kg⁻¹, sometimes in combination with low concentration of tetracyclines. One sample contained 369 µg kg⁻¹ dihydrostreptomycin, suggesting the test shows sufficient sensitivity to detect this residue at its MRL of 1000 µg kg⁻¹. However, two other samples containing 324 and 614 µg kg⁻¹ dihydrostreptomycin were not detected, so no conclusive evidence was obtained with respect to this specific aminoglycoside. The Premi® Test-kidney analysis yielded one additional MRL violation that was not observed by any of the other tests, a porcine kidney sample containing 870 µg kg⁻¹ gentamicin. The second gentamicin containing sample in this survey (593 µg kg⁻¹) was not detected by Premi® Test.

Remarkably, all samples showing combinations of tetracyclines and aminoglycosides were from calves, while all other samples containing solely aminoglycosides, concerned pigs. This striking bias prompted us to perform additional analysis on calve kidneys that appeared compliant from both the NAT-screening and the Premi® Test-kidney. This revealed that 80% of these samples contained low concentrations of tetracyclines, varying from concentrations below the limit of quantification (10 µg kg⁻¹) to 120 µg kg⁻¹, mostly oxytetracycline with traces of doxycycline.

It remains uncertain whether Premi® Test is sufficiently sensitive for accurate tetracycline detection in kidney. This study comprised several animals with muscle OTC concentrations between 41 and 82 µg kg⁻¹ that all remained unnoticed by Premi® Test-kidney. On the other hand, the only actual MRL violation (305 µg kg⁻¹ doxycycline) was effectively detected and proved that changing from muscle to kidney enhances the detection of tetracycline residues in slaughter animals.
From the results, it can be concluded that Premi® Test applied to kidney is more sensitive to aminoglycosides compared to the NAT screening. Since the manufacturer of Premi® Test does not claim detection limits for kidney, the sensitivity of the test was verified with spiked kidney and appeared to be between 250-500 µg kg\(^{-1}\) for neomycin and between 500-1000 µg kg\(^{-1}\) for gentamicin. For comparison, using muscle as a matrix, sensitivity is claimed to be 100 µg kg\(^{-1}\) for gentamicin and 300 µg kg\(^{-1}\) for neomycin. An evaluation of the applicability of the test for kidney yielded a sensitivity for gentamicin of 1500 µg kg\(^{-1}\) (Cantwell and O’Keeffe 2006). Another study showed a positive response threshold concentration for neomycin in kidney of 500-1200 µg kg\(^{-1}\) (Schneider and Lehotay 2008). From the available information it can be concluded that Premi® Test can easily detect MRL violations for neomycin in kidney, and is also capable of detecting gentamicin and dihydrostreptomycin concentrations at or close to the kidney MRL level, though more extensive validation on gentamicin and dihydrostreptomycin is required to determine whether the β-error is < 5%, in accordance to EC 2002/657 requirements (European Commission 2002).

Conclusions

Within the European Union, no harmonized approach exists with respect to screening methods for antibiotic residues. As a consequence, detection capabilities of the methods used vary widely, and the effectiveness of monitoring and surveillance therefore highly depends on the applied method. In this study we evaluated and compared the performance of the EU-four plate method, the Nouws Antibiotic Test, and Premi® Test-muscle and -kidney, by applying them to a large number to routine samples.

The most frequently occurring residue types in this study were tetracyclines and aminoglycosides, the latter only in kidney. Occurrence of specific residues however is influenced by several factors, e.g. which drugs are prescribed, administration route and subsequent residue depletion, attitude towards withdrawal times etc. and may therefore differ between countries.
Concerning the EU4pt it can be concluded that this test lacks sufficient sensitivity to be used in routine monitoring. Also the sample preparation (cutting of meat disks) is considered relatively labor-intensive. The test easily suffered from microbial contamination, which made the result sometimes difficult to interpret. Premi® Test-muscle showed a slightly better result, since it also detected the sulfamethazine MRL violation, though it suffers from a very high false-positive rate, which was already observed in the previous study (Pikkemaat et al. 2009a).

The NAT appeared the most sensitive test with respect to tetracyclines, and is capable of detecting this residue group well below its MRL. The test yields a group-specific identification, which reduces confirmatory efforts. Sample preparation for the initial screening (renal pelvis fluid) is the least laborious of the evaluated methods. A disadvantage of this matrix however, is the poor correlation with the actual concentration in kidney and muscle. This became most evident from the unnoticed gentamicin MRL violation, that was identified by the Premi® Test-kidney. Premi® Test-kidney proved to be particularly sensitive for aminoglycoside residues and it could be concluded that kidney is more suitable than muscle for detection of tetracyclines as well. However, the results on tetracycline residues obtained so far, are not sufficient to judge whether Premi® Test-kidney complies with the required sensitivity for detection of tetracyclines in slaughter animals, and an additional survey will be performed to answer this question.

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**Figure captions**

Figure 1. Flow sheet of the tetracycline screening and confirmatory procedure as applied in the Dutch national monitoring program. Between brackets the numbers related to this study.

Figure 2a. Correlation between the inhibition zone on the NAT-screening and aminoglycoside residue concentration in kidney. Neomycin is indicated by squares, dihydrostreptomycin by diamonds and gentamicin by dots. The minimum inhibition zone equals the diameter of the punch hole, which is 14 mm.

Figure 2b. Correlation between the inhibition zone on the NAT post-screening kidney and aminoglycoside residue concentration in kidney. Representation of the symbols is
similar to Fig. 2a (squares: neomycin, diamonds: dihydrostreptomycin, dots: gentamicin).

The minimum inhibition zone equals the diameter of the punch hole, which is 14 mm.
Renal pelvis fluid
Screening (735)

Suspect (24)

Negative: Compliant

Post-screening: Kidney fluid

Negative (9), or < Pos. control (7): Compliant

> pos. ctrl.
(8)

Confirmatory analysis (LC/MS²)

non-compliant result: 1
Figure 2a.
Figure 2b.

![Graph showing inhibition zone vs. concentration (µg/kg)]
Table 1. Overview of results of individual tests on non-compliant samples encountered in this study

<table>
<thead>
<tr>
<th>Non-compliant results</th>
<th>Matrix</th>
<th>MRL (µg kg(^{-1}))</th>
<th>EU4pt</th>
<th>NAT</th>
<th>Premi(^{®}) Test muscle</th>
<th>Premi(^{®}) Test kidney</th>
</tr>
</thead>
<tbody>
<tr>
<td>Sulfamethazine (1100 µg kg(^{-1}))</td>
<td>muscle</td>
<td>100</td>
<td>-</td>
<td>+</td>
<td>+</td>
<td>+</td>
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<tr>
<td>Doxycycline (305 µg kg(^{-1}))</td>
<td>muscle</td>
<td>100</td>
<td>+</td>
<td>+</td>
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<tr>
<td>Tulathromycin* (648 µg kg(^{-1}))</td>
<td>muscle</td>
<td>-</td>
<td>+</td>
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<tr>
<td>Gentamicin (870 µg kg(^{-1}))</td>
<td>kidney</td>
<td>750</td>
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<td>Neomycin (5207 µg kg(^{-1}))</td>
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* Technically this is not a non-compliant result, since no MRL has been established for tulathromycin in muscle, but since the other carcass MRL (skin plus fat) is set at 100 µg kg\(^{-1}\) we consider this sample non-compliant.
Table 2. Overview of tetracycline and aminoglycoside confirmatory results on NAT suspect samples and Premi®Test positive samples

<table>
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<tr>
<th>Sample</th>
<th>Species</th>
<th>NAT-screening diameter (mm)</th>
<th>PremiTest</th>
<th>NATpost-screening kidney diameter (mm)</th>
<th>Chemical confirmation</th>
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