



HAL
open science

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)

Mariel G Pikkemaat, Michel L B A Rapallini, Tina Zuidema, J.W. Alexander Elferink, Sabrina Oostra-van Dijk, Wilma D.M. Driessen-van Lankveld

► To cite this version:

Mariel G Pikkemaat, Michel L B A Rapallini, Tina Zuidema, J.W. Alexander Elferink, Sabrina Oostra-van Dijk, et al.. 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). *Food Additives and Contaminants*, 2010, 28 (1), pp.26. 10.1080/19440049.2010.535027 . hal-00654144

HAL Id: hal-00654144

<https://hal.science/hal-00654144>

Submitted on 21 Dec 2011

HAL is a multi-disciplinary open access archive for the deposit and dissemination of scientific research documents, whether they are published or not. The documents may come from teaching and research institutions in France or abroad, or from public or private research centers.

L'archive ouverte pluridisciplinaire **HAL**, est destinée au dépôt et à la diffusion de documents scientifiques de niveau recherche, publiés ou non, émanant des établissements d'enseignement et de recherche français ou étrangers, des laboratoires publics ou privés.



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)

Journal:	<i>Food Additives and Contaminants</i>
Manuscript ID:	TFAC-2010-299.R1
Manuscript Type:	Original Research Paper
Date Submitted by the Author:	17-Oct-2010
Complete List of Authors:	Pikkemaat, Mariel; RIKILT-Institute of food safety Rapallini, Michel; Food and Consumer Product Safety Authority Zuidema, Tina; RIKILT-Institute of food safety Elferink, J.W.; RIKILT-Institute of food safety Oostra-van Dijk, Sabrina; RIKILT-Institute of food safety Driessen-van Lankveld, Wilma; RIKILT-Institute of food safety
Methods/Techniques:	Screening - microbial screening
Additives/Contaminants:	Veterinary drug residues - antibiotics
Food Types:	Animal products – meat

SCHOLARONE™
Manuscripts

1
2
3
4 1 **Screening methods for detection of antibiotic residues in slaughter**
5
6 2 **animals: comparison of the EU-four plate method, the Nouws Antibiotic**
7
8 3 **Test and the Premi[®]Test (applied to muscle and kidney)**
9
10 4

11 Mariël G. Pikkemaat^{1*}, Michel L.B.A. Rapallini², Tina Zuidema¹, J.W. Alexander
12 Elferink¹, Sabrina Oostra-van Dijk¹, Wilma D.M. Driessen-van Lankveld¹
13
14

15
16
17 ¹RIKILT – Institute of Food Safety, Wageningen University and Research Centre,
18 P.O. Box 230, 6700 AE Wageningen, The Netherlands
19

20
21
22 ²Food and Consumer Product Safety Authority, Region East,
23 P.O. Box 144, 6700 AC Wageningen, The Netherlands
24
25

26
27
28 *Corresponding author: Marjel.pikkemaat@wur.nl
29
30
31
32
33
34
35
36
37
38
39
40
41
42
43
44
45
46
47
48
49
50
51
52
53
54
55
56
57
58
59
60

1
2
3 **Abstract**
4
5
6

7
8
9
10
11
12
13
14
15
16
17
18
19
20
21
22
23
24
25
26
27
28
29
30
31
32
33
34
35
36
37
38
39
40
41
42
43
44
45
46
47
48
49
50
51
52
53
54
55
56
57
58
59
60

16
17 Microbial growth inhibition tests are widely used as the primary screening approach for
18 the detection of antibiotic residues in slaughter animals. In this study we evaluated and
19 compared the performance of the EU-four plate method (EU4pt), the Nouws Antibiotic
20 Test (NAT), and a commercial ampoule test, the Premi[®]Test (applied to both muscle and
21 kidney), by parallel analysis of 735 slaughter animals. The EU4pt only showed
22 significant inhibition with two muscle samples containing 305 $\mu\text{g kg}^{-1}$ doxycycline and
23 648 $\mu\text{g kg}^{-1}$ tulathromycin, while an MRL violation of 1100 $\mu\text{g kg}^{-1}$ sulfamethazine
24 remained unnoticed. Premi[®]Test-muscle only detected the sulfamethazine containing
25 sample, all other (1.1%) suspect samples appeared false-positive results. The same test
26 applied to kidney yielded 4.1 % suspect samples, while the NAT-screening (based on
27 analysis of renal pelvis fluid) showed 4.9 % suspect results. The vast majority of these
28 samples contained tetracycline and/or aminoglycoside residues. Premi[®]Test-kidney
29 appeared more sensitive to aminoglycosides than the NAT-screening, which failed to
30 detect an MRL violation of 870 $\mu\text{g kg}^{-1}$ gentamicin in kidney. Detection of <MRL levels
31 of tetracycline residues by the NAT proved its suitability for this residue group. Whether
32 Premi[®]Test is sufficiently sensitive for accurate tetracycline detection in kidney remained
33 doubtful, although changing over to kidney definitely improved the suitability of
34 Premi[®]Test for detection of residues in slaughter animals.
35

36 **Keywords:** antibiotic residues; microbial screening method; Premi[®]Test; Nouws
37 Antibiotic Test; EU-four plate test

38 Introduction

39

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

47

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

57

58 Large scale screening of animal products for the presence of residues of antibiotics is
59 commonly applied using microbial screening methods. These methods are based on
60 bacterial growth inhibition of a sensitive test-bacterium, and essentially originate from
61 procedures testing pharmaceutical preparations and body fluids (Grove and Randall
62 1955). In contrast to the field of the instrumental confirmatory methods, where major
63 efforts are spend on method development, establishing EU Maximum Residue Limits has
64 induced only minor efforts on method enhancement and implementation of microbial
65 screening methods. It has been shown that the current state-of-the-rt is often not sufficient
66 to support legislative requirements (Pikkemaat 2009; Pikkemaat et al. 2009a). Since these
67 methods form the foundation of the residue monitoring system, this situation leads to
68 inadequate consumer protection. In order to draw more attention to this problem, we

69 evaluated the performance of two commonly used screening methods and compared them
70 with the method routinely applied in the Netherlands, the Nouws Antibiotic Test (NAT)
71 (Pikkemaat et al. 2008; Pikkemaat et al. 2009b).

72
73 First of all the EU four-plate method (EU4pt) was assessed. This method was first
74 published in 1980 (Bogaerts and Wolf 1980) and is based on three *Bacillus subtilis* based
75 test plates and a fourth *Micrococcus luteus* plate (ATCC 9341, renamed to *Kocuria*
76 *rhizophila* (Tang and Gillevet 2003). Within the EU this method served as a reference
77 until it was decided to determine acceptable residue limits on a more scientifically based
78 approach (the effectuation of Council Regulation (EEC) 2377/90 (European Commission
79 1990)) and MRLs were set at levels beyond the detection capability of this method.
80 Although the EU4pt is generally recognized not to be sufficiently sensitive, it is still used
81 in many laboratories (Berendsen et al. 2010; Gaudin et al. 2008).

82
83 As an example of an ampoule or tube test, Premi[®]Test (DSM) was included. This type of
84 test is based on *Bacillus stearothermophilus*, a bacterium particularly sensitive to beta-
85 lactam antibiotics. Recently the Premi[®]Test and a similar test, the Explorer (Zeu-
86 Immunotec), have gone through an extensive validation carried out by the EU Community
87 reference laboratory (Gaudin et al. 2008, Gaudin et al. 2009). In particular with respect to
88 tetracyclines, it has been shown that Premi[®]Test lacks sufficient sensitivity to detect these
89 antibiotics at the MRL in muscle (Pikkemaat et al. 2009a; Okerman et al. 2004).

90 According to the manufacturer DSM, Premi[®]Test is also suitable for other matrices,
91 including kidney. Since for tetracyclines (and several other antibiotics) the MRL in
92 kidney tissue is much higher (tetracyclines: 100 $\mu\text{g kg}^{-1}$ in muscle, vs. 600 $\mu\text{g kg}^{-1}$ in
93 kidney), this organ could be a more suitable matrix for antibiotic screening. To
94 investigate the potential of applying kidney instead of muscle, both matrices were tested
95 in parallel.

96
97 The Nouws Antibiotic Test (NAT) comprises a 5-plate (residue group-specific) initial
98 screening based on the analysis of renal pelvis-fluid (Pikkemaat et al. 2008), and two
99 subsequent post-screening tests for further analysis of kidney and/or muscle of suspect

1
2
3 100 animals (Pikkemaat et al. 2009a; Pikkemaat et al. 2009b). The EU4pt, the NAT and the
4
5 101 Premi[®]Test (muscle and kidney), were performed in parallel on slaughter animals tested
6
7 102 within the framework of the national monitoring program.
8

9 103

10 104 **Materials and Methods**

11 105 *Sample material*

12 106 All animal samples analyzed in this study were taken as part of the national monitoring
13
14 107 program. After slaughter, one of the kidneys and a piece of lean muscle were transported
15
16 108 cool to the laboratory and analyzed the next day. During the months of June and October
17
18 109 2009, a total of 491 pigs, 156 calves, 75 cows, 9 sheep, two goats and two horses (a total
19
20 110 of 735 animals) were subjected to the three parallel tests.
21
22

23 111

24 112 *Methods*

25 113 *Nouws Antibiotic Test*

26 114 The Nouws Antibiotic Test is a test system involving an initial screening of renal pelvis
27
28 115 fluid (pre-urine) and post-screening of muscle and kidney. Initial and post-screening tests
29
30 116 each comprise a series of test plates, each optimized for the detection of one or two
31
32 117 antibiotic groups in a specific matrix. The initial screening comprises five plates: a
33
34 118 *Bacillus cereus* ATCC 1178 plate specific for tetracyclines (T), a *Kocuria rhizophila*
35
36 119 ATCC 9341 plate specific for beta-lactam antibiotics and macrolides (B&M), a *Yersinia*
37
38 120 *ruckeri* NCIM 13282 plate specific for quinolones (Q), a *Bacillus pumilus* CN 607 plate
39
40 121 specific for sulfonamides & diaminopyrimidines (S) and a *Bacillus subtilis* BGA plate
41
42 122 specific for aminoglycosides (A). The exact composition of the individual test plates and
43
44 123 the procedure were described in detail in (Pikkemaat et al. 2008). In brief, an incision is
45
46 124 made in the kidney and renal pelvis is collected by absorption to paper disks, which are
47
48 125 placed on the interface of the medulla and the cortex. Each kidney is sampled with five
49
50 126 paper disks, one for each test-plate. The paper disks are applied to punch holes in the test
51
52 127 plate and supplemented with a plate specific buffer. After overnight incubation the
53
54 128 emergence of a growth inhibition zone indicates the presence of antimicrobial residues in
55
56 129 the animal.
57

58 130
59
60

1
2
3 131 Suspect samples, showing an inhibition zone on one or more test plates, are additionally
4
5 132 analyzed by post-screening of kidney and/or muscle, limited to the residue group for
6
7 133 which the initial screening tested positive. Samples for post-screening are prepared by
8
9 134 homogenizing kidney or muscle and isolating tissue fluid from the homogenate by
10
11 135 centrifugation after a brief heating step. The post-screening is based on a multi-plate
12
13 136 principle similar to the initial screening and described in detail in Pikkemaat et al.
14
15 137 (2009b) for kidney and in Pikkemaat et al. (2009a) for muscle.
16

17 138

18 139 *EU four-plate test*

19 140 The EU4pt was performed essentially similar to Bogaerts and Wolf (1980). Test agar pH
20
21 141 6 (Merck), Antibiotic medium II (Difco) (adjusted to pH 7.2, supplemented with
22
23 142 trimethoprim to a final concentration of 50 $\mu\text{g l}^{-1}$), and Test agar pH 8 (Merck), were
24
25 143 inoculated with approximately 10^4 CFU/ml *B. subtilis* BGA spores (Merck) and Test agar
26
27 144 pH 8 (Merck) was inoculated with 10^4 CFU ml⁻¹ *K. rhizophila* ATCC 9341. A volume of
28
29 145 105 ml of the inoculated growth media was poured in 245 x 245 mm square petri dishes,
30
31 146 resulting in a layer of 2 mm thickness. Samples were prepared by freezing a thin slice of
32
33 147 muscle briefly at -80°C and subsequently take out disks, using a cork borer with a
34
35 148 diameter of 4 mm. Meat disks were placed on each of the four test plates, with a
36
37 149 maximum of 24 samples per plate. As a quality control on each plate a paper disk
38
39 150 impregnated with either 10 IE penicillin, 5 μg sulfamethazine, 0.5 μg
40
41 151 dihydrostreptomycin or 0.5 μg tylosin was added (by adding 100 μl of a fresh 10x stock).
42
43 152 Test plates were incubated for 14-16 hr at 30°C (*B. subtilis*) or 37°C (*K. rhizophila*).
44

45 153

46 154 *Premi[®]Test*

47 155 The Premi[®]Test was essentially performed according to the manufacturers instructions.
48
49 156 The test is based on the analysis of 100 μl of liquid sample extracted from the tissue. For
50
51 157 the preparation of kidney juice, a 10 ml centrifuge tube was filled with roughly cut pieces
52
53 158 of kidney taken from the cortex-medulla interface. This sample was heated for 10 min. at
54
55 159 80°C, then cooled down and centrifuged for 10 min at 27000 x g. Sample preparation for
56
57 160 muscle was performed in a similar way. Samples of 100 μl of supernatant were applied
58
59 161 on the test vials and removed after 20-30 minutes of pre-incubation at room temperature.
60

1
2
3 162 The vials were sealed and transferred to a 64°C water bath and incubated until the
4
5 163 majority of the samples had turned yellow, which was usually after approximately 3 hrs
6
7 164 with muscle fluid and 4 hrs when kidney was analyzed. Samples showing a positive
8
9 165 result, which was defined as absence of any color change, were retested the next day,
10
11 166 including a penicillinase test for identification of beta lactam antibiotics. Only samples
12
13 167 also positive in this second test were forwarded for chemical confirmation.
14

168

169 *Chemical confirmation*

170 Samples showing a positive (suspect) result in one of the tests, but for which the group
171 identity could not be determined from the microbial screening result, were first analyzed
172 using a multi-compound screening method based on high-resolution liquid
173 chromatography combined with time-of-flight mass spectrometry (HRLC-ToF-MS)
174 (Peters et al. 2009). In short, samples were extracted intensive for 30 min using a mixture
175 of acetonitrile and water (6/4; v/v), after which the samples were centrifuged (15 min;
176 3600 g; 10°C). An aliquot of the supernatant was diluted 20 times using water and
177 applied to a StrataX SPE column (60 mg). After elution using a methanol/acetonitrile
178 mixture (1/1; v/v), the eluate was evaporated under a stream of nitrogen at 40°C till near
179 dryness and the residue was re-dissolved in acetonitrile. After addition of 0.1% formic
180 acid the extract was analyzed by HRLC-ToF-MS in the full scan mode. Chromatographic
181 separation was achieved using a Waters Acquity UPLC system equipped with a reversed
182 phase Waters Acquity UPLC BEH C₁₈ analytical column (100 x 2.1 mm; 1.7µm) using a
183 0.1% formic acid/acetonitrile gradient. The mass spectrometer was operated in the
184 electrospray positive mode using a mass range of 100 – 1000 Da.
185

186

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

1
2
3 192 based on liquid-liquid extraction followed by solid-phase extraction or an ultrafiltration
4
5 193 step.

6
7 194

8
9 195

10 196 **Results and discussion**

11 197 *General*

12
13
14 198 The Nouws Antibiotic Test (NAT) is the routine antibiotic screening method applied to
15
16 199 slaughter animals in the Netherlands. During the months of June and October 2009 all
17
18 200 animals which were analyzed within the framework of the national monitoring program,
19
20 201 were tested additionally with the EU4pt and the Premi[®]Test, the latter applied both on
21
22 202 muscle and kidney. In total 735 animals (pigs, bovines, sheep, goats and horses) were
23
24 203 screened. It was anticipated that the number of treated animals would be higher during
25
26 204 October, because deteriorating weather conditions potentially would cause increased
27
28 205 health problems, but this was not reflected in the numbers of suspect animals. The
29
30 206 percentage was even slightly higher in June: out of 405 animals 5.2 % and 4.2% yielded a
31
32 207 suspect result with the NAT and Premi[®]Test-kidney respectively, while in October 4.5%
33
34 208 resp. 3.9% suspect results were found among the remaining 330 animals.

35
36 209

37 210 Out of the 735 animals, only four showed an MRL violation, all of which were pigs. Two
38
39 211 pigs showed muscle concentrations of respectively 305 $\mu\text{g kg}^{-1}$ doxycycline and 1100 μg
40
41 212 kg^{-1} sulfamethazine. The other MRL violations were found in porcine kidneys, containing
42
43 213 870 $\mu\text{g kg}^{-1}$ gentamicin and 5207 $\mu\text{g kg}^{-1}$ neomycin. Also an animal containing a
44
45 214 significant level (648 $\mu\text{g kg}^{-1}$) of tulathromycin in muscle was identified, but for this
46
47 215 compound no MRL has been established in muscle, so statutorily it could not be
48
49 216 considered an MRL violation. In some countries however a zero-tolerance policy is
50
51 217 applied, though this remains disputable because it concerns a registered drug with a set
52
53 218 acceptable daily intake (ADI). An overview of the results of the individual tests on these
54
55 219 samples is shown in Table 1.

56
57 220

58 221 Compared to a previous study comprising 591 animals, in which we compared the
59
60 222 performance of the NAT, the Screening Test for Antibiotic Residues (STAR) and

1
2
3 223 Premi[®]Test applied to muscle, the percentage of MRL violations is somewhat lower in
4 224 the current survey (0.54 vs. 0.67%), while in the previous study we did not yet have the
5
6 225 possibility for confirmation of the aminoglycosides, which may even have led to an
7
8 226 underestimation of the number of MRL violations.
9

10 227

11
12 228 [Insert Table 1 about here]

13 229

14 230 *The EU-four plate test*

15 231

16 232 Evaluating the results of the individual tests, it can be concluded that the EU4pt yielded a
17
18 233 very low number of suspect samples. This test is based on the analysis of muscle discs,
19
20 234 and subsequently is not suited for detecting MRL violations in kidney. Significant
21
22 235 inhibition zones were observed with only two samples. The presence of 305 $\mu\text{g kg}^{-1}$
23
24 236 doxycycline became apparent from all three *B. subtilis* plates, with the largest inhibition
25
26 237 zone (23 mm) on the pH 6 plate. The tulathromycin sample ($648 \mu\text{g kg}^{-1}$) caused an
27
28 238 inhibition zone of 23 mm on the *K. rhizophila* plate. The sulfamethazine containing
29
30 239 sample was not detected, even though the concentration in the sample was > 10 times the
31
32 240 MRL.
33

34 241

35
36 242 Occasionally it appeared difficult to judge the result of this test, since it easily suffered
37
38 243 from microbial contamination causing growth on the surface of the plate around the meat
39
40 244 disk. This sometimes was attended by growth inhibition in the agar. Some of these
41
42 245 questionable results were additionally analyzed by the multi compound ToF-MS
43
44 246 confirmation method but always turned out to be negative.
45

46 247

47 248 *The Nouws Antibiotic Test*

48
49 249 The NAT yielded a total of 36 suspect results (4.9%) with the initial screening of renal
50
51 250 pelvis fluid. The test comprises 5 individual test plates, each one preferably sensitive to
52
53 251 one or two groups of antibiotic residues. Of these 36 samples, one sample was
54
55 252 specifically detected on the sulfonamide test plate (originating from the animal containing
56
57 253 $1100 \mu\text{g kg}^{-1}$ sulfamethazine in its muscle), 24 were suspect on the tetracycline specific
58
59
60

1
2
3
4 254 plate and another 11 on the aminoglycoside test plate. The tulathromycin containing
5 255 sample showed some inhibition on the aminoglycoside test plate, but was primarily
6
7 256 detected (showing a larger inhibition zone) on the macrolide & beta-lactam test plate.
8
9 257 Compared to the previous study, the percentage of tetracycline suspect animals decreased
10
11 258 slightly (4.9 to 3.3 %) while the percentage of aminoglycoside suspect samples was
12
13 259 nearly identical (1.5 vs. 1.4%).

14 260

15
16 261 Samples suspect for tetracyclines in the initial screening were subjected to post-screening
17
18 262 of the kidney, which in routine analysis is followed by chemical confirmation in muscle
19
20 263 (figure 1). It has been shown before that the kidney post-screening result is a more
21
22 264 reliable indicator for the concentration in muscle than the initial (renal pelvis fluid based)
23
24 265 screening result (Pikkemaat et al. 2009a). This is presumably caused by variations in the
25
26 266 renal pelvis fluid available for absorption to the paper disk. Nine (37.5%) of these
27
28 267 initially suspect samples were found negative in post screening kidney, while another
29
30 268 seven (29%) showed an inhibition zone smaller than the control sample of $600 \mu\text{g kg}^{-1}$
31
32 269 oxytetracycline (OTC), which in the routine procedure is the cut-off for further chemical
33
34 270 confirmation. So in practice only muscle samples of the remaining eight animals would
35
36 271 be further analyzed by LC-MS/MS, though for the purpose of this study and to verify the
37
38 272 assumption that this procedure does not yield false-compliant results, all animals showing
39
40 273 a suspect result in the screening were fully analyzed.

41 274

42 275 [Insert Figure 1 about here]

43 276

44 277 With regard to samples showing no inhibition in the post-screening kidney,
45
46 278 concentrations in the muscle were found to be always less than $26 \mu\text{g kg}^{-1}$ (doxycycline),
47
48 279 while three of them were even below the limit of detection of the method (Table 2). For
49
50 280 those that showed inhibition, but below the cut-off for chemical confirmation,
51
52 281 concentrations were usually between 20 and $30 \mu\text{g kg}^{-1}$, with a maximum found at $42 \mu\text{g}$
53
54 282 kg^{-1} doxycycline. Tetracycline concentrations in samples which according to the routine
55
56 283 procedure would be forwarded for chemical confirmation, started from minimum levels

1
2
3 284 of 25 $\mu\text{g kg}^{-1}$ doxycycline and 41 $\mu\text{g kg}^{-1}$ OTC. Only one out of the eight samples
4
5 285 appeared to be a true non-compliant result, containing 305 $\mu\text{g kg}^{-1}$ doxycycline.
6
7 286

8
9 287 From these results we can conclude that the procedure efficiently downsizes the number
10
11 288 of samples requiring chemical confirmation to approximately one-third of the original
12
13 289 suspect samples, without resulting in false-compliant results. It should be noted that the
14
15 290 majority of the tetracycline suspect samples contained doxycycline, either alone or in
16
17 291 combination with OTC, only in four samples OTC was the sole tetracycline residue
18
19 292 present. This could of course be attributed to a bias caused by the method, which is more
20
21 293 sensitive to doxycycline, however taking into account the monitoring results obtained
22
23 294 since the introduction of the NAT in 2004, a clear overall shift from OTC to doxycycline
24
25 295 residues is observed (M. Rapallini, unpublished results).
26
27 296

28
29 297 The other major residue group observed by the NAT-screening, were the
30
31 298 aminoglycosides. Nine out of the 10 samples that were suspect for this group based on
32
33 299 the renal pelvis screening, also showed an inhibition zone in the kidney post-screening.
34
35 300 The one sample negative sample also appeared negative after LC-MS/MS analysis and
36
37 301 should be considered as a false-positive result. Aminoglycoside residue levels in kidney
38
39 302 ranged from 106-5027 $\mu\text{g kg}^{-1}$ neomycin (the highest concentration being an MRL
40
41 303 violation), 324-614 $\mu\text{g kg}^{-1}$ dihydrostreptomycin (MRL in kidney: 1000 $\mu\text{g kg}^{-1}$) and 593
42
43 304 $\mu\text{g kg}^{-1}$ gentamicin (Table 2). The latter yielded an NAT-screening inhibition zone of 26
44
45 305 mm, which is quite remarkable, since the second aminoglycoside MRL violation, 870 μg
46
47 306 kg^{-1} gentamicin, was not noticed by the NAT-screening. This can be explained from the
48
49 307 poor correlation between screening and final concentration in kidney and could only be
50
51 308 prevented by direct post-screening analysis of the kidney, for which the correlation is
52
53 309 much better. This is illustrated in Figure 2, showing screening and post-screening results
54
55 310 of aminoglycoside positive samples.
56
57 311

58
59 312 [Insert Figure 2 about here]
60
61 313

1
2
3 314 In practice so far no cut-off (analogous to the tetracycline procedure) has been
4
5 315 determined, beyond which a sample should be forwarded for chemical quantification. The
6
7 316 current kidney post-screening results suggest a cut-off inhibition zone around 30 mm,
8
9 317 which is somewhat larger than the original validation study suggested (Pikkemaat et al.
10
11 318 2009b), but more data should be collected to enable a reliable estimation.
12

13
14 320 Additional analysis of the muscle of the suspect animals showed that aminoglycoside
15
16 321 residue levels in muscle were negligible. Only a single animal with a neomycin level of
17
18 322 $3973 \mu\text{g kg}^{-1}$ in kidney also showed traces of this residue in muscle ($<5 \mu\text{g kg}^{-1}$). The
19
20 323 aminoglycoside residue group therefore is a very pronounced example of a non-compliant
21
22 324 result in kidney not necessarily reflecting a non-compliant result in muscle. This
23
24 325 observation implies a serious discrepancy between the outcome of control/surveillance
25
26 326 strategies based on the analysis of either kidney or muscle.
27

28 328

29 329 *Premi[®] Test-muscle*

30
31 330 Premi[®]Test applied to non-contaminated tissue should result in a color change from
32
33 331 purple to yellow, and the absence or delay in color change indicates the presence of a
34
35 332 growth inhibiting compound. In this study, only samples that showed a complete absence
36
37 333 of color change were considered suspect. The number of samples that would need further
38
39 334 testing in case all samples showing intermediate results were also considered,
40
41 335 occasionally was 5 to 10-fold more, and was considered not suitable in large scale
42
43 336 screening. Most of these intermediate results probably should be attributed to matrix
44
45 337 variability. Suspect samples were always retested in presence and absence of
46
47 338 penicillinase for confirming the presence of beta-lactam antibiotics.
48

49
50 340 Only 9 muscle samples tested positive with the Premi[®]Test, and none of these suspect
51
52 341 results were attributable to the presence of beta-lactam antibiotics. One of the positive
53
54 342 results concerned the sample containing $1100 \mu\text{g kg}^{-1}$ sulfamethazine, but in all other
55
56 343 samples the presence of antibiotic residues could not be confirmed, resulting in a very
57
58 344 high false-positive rate of the suspect samples. Among these false-positives was one
59
60

1
2
3 345 bovine (non-calve) sample, all others were porcine samples. The doxycycline ($305 \mu\text{g kg}^{-1}$)
4 346 ¹) MRL violation was not detected, once more establishing that Premi[®]Test applied to
5
6 347 muscle is not suitable for effective tetracycline detection. Remarkably also the sample
7
8 348 containing $648 \mu\text{g kg}^{-1}$ tulathromycin remained undetected, while in our previous study a
9
10 349 sample containing $156 \mu\text{g kg}^{-1}$ tulathromycin was found suspect (Pikkemaat et al. 2009a).
11
12 350

14 351 *Premi[®]Test-kidney*

15 352 The number of kidney samples yielding a positive Premi[®]Test result was substantially
16
17 353 higher. One of the main problems generally associated with using kidney as a matrix, is
18
19 354 the occurrence of false-positive results, probably caused by the action of enzymes present
20
21 355 in this matrix, generally referred to as “natural growth inhibiting compounds”. A
22
23 356 common way to circumvent this (an approach generally applied in milk analysis) is to
24
25 357 inactivate these natural growth inhibiting compounds by heating the sample. Using
26
27 358 Premi[®]Test for analysis of kidney, the manufacturer advises the incorporation of a heat-
28
29 359 pretreatment step (10 min at 80°C) after applying the kidney juice to the ampoule.
30
31 360 However, in our hands this approach appeared to cause problems, since the
32
33 361 heating/inactivation step caused a strong coloring of the growth medium. This made the
34
35 362 ultimate color change hard to assess, and also considerably increased the required
36
37 363 incubation time. This problem could however easily be circumvented by performing the
38
39 364 heating step before applying the sample to the tube.
40
41 365

42 366 Similar to muscle analysis samples were considered positive only when there was
43
44 367 complete absence of color change. Suspect results were first verified with a second
45
46 368 analysis including penicillinase, however none of the kidneys appeared suspect for beta-
47
48 369 lactam antibiotics. Applying the Premi[®]Test to kidney yielded 30 suspect animals. While
49
50 370 this number is similar to the number of suspect samples obtained after the NAT screening
51
52 371 (36), the overlap of samples suspect in both tests was limited to 8 animals. These
53
54 372 included the sulfamethazine and doxycycline MRL violations, and a tetracycline suspect
55
56 373 animal which was confirmed to contain $34 \mu\text{g kg}^{-1}$ doxycycline in the muscle (the
57
58 374 concentration in kidney was not determined). Furthermore the tulathromycin containing
59
60 375 sample, and several aminoglycoside suspect samples were identified by both methods.

1
2
3 376

4
5 377 In twelve suspect samples the presence of antibiotic residues could not be confirmed and
6
7 378 these should therefore be considered false-positive results (40%), notably all of these
8
9 379 were pig samples. One sample contained $15 \mu\text{g kg}^{-1}$ tylosin, but considering the reported
10
11 380 sensitivity for muscle, which is $50\text{-}100 \mu\text{g kg}^{-1}$, it seems somewhat unlikely that this low
12
13 381 concentration actually caused the inhibition. All other suspect samples appeared to
14
15 382 contain aminoglycoside residues (Table 2). Most of them contained neomycin, with
16
17 383 concentrations starting from around $470 \mu\text{g kg}^{-1}$, sometimes in combination with low
18
19 384 concentration of tetracyclines. One sample contained $369 \mu\text{g kg}^{-1}$ dihydrostreptomycin,
20
21 385 suggesting the test shows sufficient sensitivity to detect this residue at its MRL of 1000
22
23 386 $\mu\text{g kg}^{-1}$. However, two other samples containing 324 and $614 \mu\text{g kg}^{-1}$
24
25 387 dihydrostreptomycin were not detected, so no conclusive evidence was obtained with
26
27 388 respect to this specific aminoglycoside. The Premi[®]Test-kidney analysis yielded one
28
29 389 additional MRL violation that was not observed by any of the other tests, a porcine
30
31 390 kidney sample containing $870 \mu\text{g kg}^{-1}$ gentamicin. The second gentamicin containing
32
33 391 sample in this survey ($593 \mu\text{g kg}^{-1}$) was not detected by Premi[®]Test.

34

35 393 Remarkably, all samples showing combinations of tetracyclines and aminoglycosides
36
37 394 were from calves, while all other samples containing solely aminoglycosides, concerned
38
39 395 pigs. This striking bias prompted us to perform additional analysis on calve kidneys that
40
41 396 appeared compliant from both the NAT-screening and the Premi[®]Test-kidney. This
42
43 397 revealed that 80% of these samples contained low concentrations of tetracyclines, varying
44
45 398 from concentrations below the limit of quantification ($10 \mu\text{g kg}^{-1}$) to $120 \mu\text{g kg}^{-1}$, mostly
46
47 399 oxytetracycline with traces of doxycycline.

48

49 401 It remains uncertain whether Premi[®]Test is sufficiently sensitive for accurate tetracycline
50
51 402 detection in kidney. This study comprised several animals with muscle OTC
52
53 403 concentrations between 41 and $82 \mu\text{g kg}^{-1}$ that all remained unnoticed by Premi[®]Test-
54
55 404 kidney. On the other hand, the only actual MRL violation ($305 \mu\text{g kg}^{-1}$ doxycycline) was
56
57 405 effectively detected and proved that changing from muscle to kidney enhances the
58
59 406 detection of tetracycline residues in slaughter animals.

407

408 From the results, it can be concluded that Premi[®]Test applied to kidney is more sensitive
409 to aminoglycosides compared to the NAT screening. Since the manufacturer of
410 Premi[®]Test does not claim detection limits for kidney, the sensitivity of the test was
411 verified with spiked kidney and appeared to be between 250-500 $\mu\text{g kg}^{-1}$ for neomycin
412 and between 500-1000 $\mu\text{g kg}^{-1}$ for gentamicin. For comparison, using muscle as a matrix,
413 sensitivity is claimed to be 100 $\mu\text{g kg}^{-1}$ for gentamicin and 300 $\mu\text{g kg}^{-1}$ for neomycin. An
414 evaluation of the applicability of the test for kidney yielded a sensitivity for gentamicin of
415 1500 $\mu\text{g kg}^{-1}$ (Cantwell and O’Keeffe 2006). Another study showed a positive response
416 threshold concentration for neomycin in kidney of 500-1200 $\mu\text{g kg}^{-1}$ (Schneider and
417 Lehotay 2008). From the available information it can be concluded that Premi[®]Test can
418 easily detect MRL violations for neomycin in kidney, and is also capable of detecting
419 gentamicin and dihydrostreptomycin concentrations at or close to the kidney MRL level,
420 though more extensive validation on gentamicin and dihydrostreptomycin is required to
421 determine whether the β -error is $< 5\%$, in accordance to EC 2002/657 requirements
422 (European Commission 2002).

423

424

425 **Conclusions**

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

432

433 The most frequently occurring residue types in this study were tetracyclines and
434 aminoglycosides, the latter only in kidney. Occurrence of specific residues however is
435 influenced by several factors, e.g. which drugs are prescribed, administration route and
436 subsequent residue depletion, attitude towards withdrawal times etc. and may therefore
437 differ between countries.

1
2
3 438

4 439 Concerning the EU4pt it can be concluded that this test lacks sufficient sensitivity to be
5 used in routine monitoring. Also the sample preparation (cutting of meat disks) is
6 440 considered relatively labor-intensive. The test easily suffered from microbial
7 441 contamination, which made the result sometimes difficult to interpret. Premi[®]Test-muscle
8 442 showed a slightly better result, since it also detected the sulfamethazine MRL violation,
9 443 though it suffers from a very high false-positive rate, which was already observed in the
10 444 previous study (Pikkemaat et al. 2009a).
11 445
12 446

13 447 The NAT appeared the most sensitive test with respect to tetracyclines, and is capable of
14 448 detecting this residue group well below its MRL. The test yields a group-specific
15 449 identification, which reduces confirmatory efforts. Sample preparation for the initial
16 450 screening (renal pelvis fluid) is the least laborious of the evaluated methods. A
17 451 disadvantage of this matrix however, is the poor correlation with the actual concentration
18 452 in kidney and muscle. This became most evident from the unnoticed gentamicin MRL
19 453 violation, that was identified by the Premi[®]Test-kidney. Premi[®]Test-kidney proved to be
20 454 particularly sensitive for aminoglycoside residues and it could be concluded that kidney
21 455 is more suitable than muscle for detection of tetracyclines as well. However, the results
22 456 on tetracycline residues obtained so far, are not sufficient to judge whether Premi[®]Test-
23 457 kidney complies with the required sensitivity for detection of tetracyclines in slaughter
24 458 animals, and an additional survey will be performed to answer this question.
25 459
26 460

27 461 **References**

28 462 Anonymous. 2010. Guidelines for the validation of screening methods for residues of
29 463 veterinary medicines, drafted by the Community Reference Laboratories Residues
30 464 (CRLs), version 20/1/2010. Available from:
31 465 http://ec.europa.eu/food/food/chemicalsafety/residues/Guideline_Validation_Screening_e
32 466 [n.pdf](#)
33 467

- 1
2
3 468 Berendsen BJA, van Rhijn JA. 2006. Residue analysis of tetracyclines in poultry muscle:
4 Shortcomings revealed by a proficiency test. *Food Addit Contam.* 23:1141-1148.
5 469
6 470
7
8 471 Berendsen BJ, Pikkemaat MG, Stolker AAM. 2010. Are antibiotic screening approaches
9 sufficiently adequate? A proficiency test. *Anal Chim Acta.* submitted.
10 472
11 473
12 474 Bogaerts R, Wolf F. 1980. A standardized method for the detection of residues of
13 antibacterial substances in fresh meat. *Fleischwirtschaft.* 60: 672–674.
14 475
15 476
16 477 Cantwell H, O'Keeffe M. 2006. Evaluation of the Premi[®]Test and comparison with the
17 One-Plate Test for the detection of antimicrobials in kidney. *Food Addit Contam.* 23:120-
18 125.
19 479
20 480
21 481 European Commission. 1990. Council Regulation (EEC) 2377/90 of 26 June 1990 laying
22 down a Community procedure for the establishment of maximum residue limits of
23 veterinary medicinal products in foodstuffs of animal origin. *Off J Eur Union.* L224:1-8.
24 482
25 483
26 484
27 485 European Commission. 1996. Council Directive 96/23/EC of 29 April 1996 on measures
28 to monitor certain substances and residues thereof in live animals and animal products
29 repealing Directives 85/358/EEC and 86/469/EEC and Decisions 89/187/EEC and
30 91/664/EEC. *Off J Eur Union.* L125:10–32.
31 486
32 487
33 488
34 489
35 490 European Commission. 2002. Commission Decision 2002/657/EC of 12 August 2002
36 implementing Council Directive 96/23/EC concerning the performance of analytical
37 methods and the interpretation of results. *Off J Eur Union.* L221:8–36.
38 491
39 492
40 493
41 494 Gaudin V, Juhel-Gaugain M, Morétain JP, Sanders P. 2008. AFNOR validation of
42 Premi[®]Test, a microbiological-based screening tube-test for the detection of antimicrobial
43 residues in animal muscle tissue. *Food Addit Contam.* 25:1451-1464.
44 495
45 496
46 497
47
48
49
50
51
52
53
54
55
56
57
58
59
60

- 1
2
3 498 Gaudin V, Hedou C, Rault A, Sanders P, Verdon E. 2009. Comparative study of three
4 screening tests, two microbiological tube tests, and a multi-sulphonamide ELISA kit for
5 499 the detection of antimicrobial and sulphonamide residues in eggs. Food Addit Contam.
6 500 26:427-440.
7 501
8 502
9
10 503 Grove DC, Randall WA. 1955. Assay methods of antibiotics, a laboratory manual. New
11 York: Medical Encyclopedia, Inc..
12 504
13 505
14 506 Okerman L, Croubels S, Cherlet M, De Wasch K, De Backer P, Van Hoof J. 2004.
15 Evaluation and establishing the performance of different screening tests for tetracycline
16 507 residues in animal tissues. Food Addit Contam. 21:145-153.
17 508
18 509
19 510 Peters RJ, Bolck YJ, Rutgers P, Stolker AAM, Nielen MWF. 2009. Multi-residue
20 screening of veterinary drugs in egg, fish and meat using high-resolution liquid
21 511 chromatography accurate mass time-of-flight mass spectrometry. J Chromatogr A.
22 512 1216:8206-8016.
23 513
24 514
25 515 Pikkemaat MG, Oostra-van Dijk S, Schouten S, Rapallini M, van Egmond HJ. 2008. A
26 new microbial screening method for the detection of antimicrobial residues in slaughter
27 516 animals: The Nouws antibiotic test (NAT-screening). Food Control. 19:781-789.
28 517
29 518
30 519 Pikkemaat MG. 2009. Microbial screening methods for detection of antibiotic residues in
31 slaughter animals. Anal Bioanal Chem. 395:893-905.
32 520
33 521
34 522 Pikkemaat MG, Rapallini MLBA, Oostra-van Dijk S, Elferink JWA. 2009a. Comparison
35 of three microbial screening methods for antibiotics using routine monitoring samples.
36 523 Anal Chim Acta. 637:298-304.
37 524
38 525
39 526 Pikkemaat MG, Oostra-Van Dijk S, Schouten J, Rapallini M, Kortenhoeven L, van
40 527 Egmond HJ. 2009b. Nouws antibiotic test: Validation of a post-screening method for
41 528 antibiotic residues in kidney. Food Control. 20:771-777.
42
43
44
45
46
47
48
49
50
51
52
53
54
55
56
57
58
59
60

1
2
3 529

4
5 530 Schneider MJ, Lehotay SJ. 2008. A comparison of the FAST, Premi[®] and KIS[™] tests for
6
7 531 screening antibiotic residues in beef kidney juice and serum. Anal Bioanal Chem.
8
9 532 390:1775-1779.

10 533

11
12 534 Tang SJ, Gillevet PM. 2003. Reclassification of ATCC 9341 from *Micrococcus luteus* to
13
14 535 *Kocuria rhizophila*. Int J Syst Evol Microbiol. 53:995-997.

15 536

16
17 537 van Holthoon FL, Essers ML, Mulder PJ, Stead SL, Caldow M, Ashwin HM, Sharman
18
19 538 M. 2009. A generic method for the quantitative analysis of aminoglycosides (and
20
21 539 spectinomycin) in animal tissue using methylated internal standards and liquid
22
23 540 chromatography tandem mass spectrometry. Anal Chim Acta. 637:135-143.

24 541

25
26 542 van Rhijn JA, Lasaroms JJP, Berendsen BJA, Brinkman UATh. 2002. Liquid
27
28 543 chromatographic-tandem mass spectrometric determination of selected sulphonamides in
29
30 544 milk. J Chromatogr A. 960:121-133.

31 545

32 546

33 547 **Figure captions**

34 548

35
36
37 549 Figure 1. Flow sheet of the tetracycline screening and confirmatory procedure as applied
38
39 550 in the Dutch national monitoring program. Between brackets the numbers related to this
40
41 551 study.

42 552

43
44
45 553 Figure 2a. Correlation between the inhibition zone on the NAT-screening and
46
47 554 aminoglycoside residue concentration in kidney. Neomycin is indicated by squares,
48
49 555 dihydrostreptomycin by diamonds and gentamicin by dots. The minimum inhibition zone
50
51 556 equals the diameter of the punch hole, which is 14 mm.

52 557

53
54
55 558 Figure 2b. Correlation between the inhibition zone on the NAT post-screening kidney and
56
57 559 aminoglycoside residue concentration in kidney. Representation of the symbols is

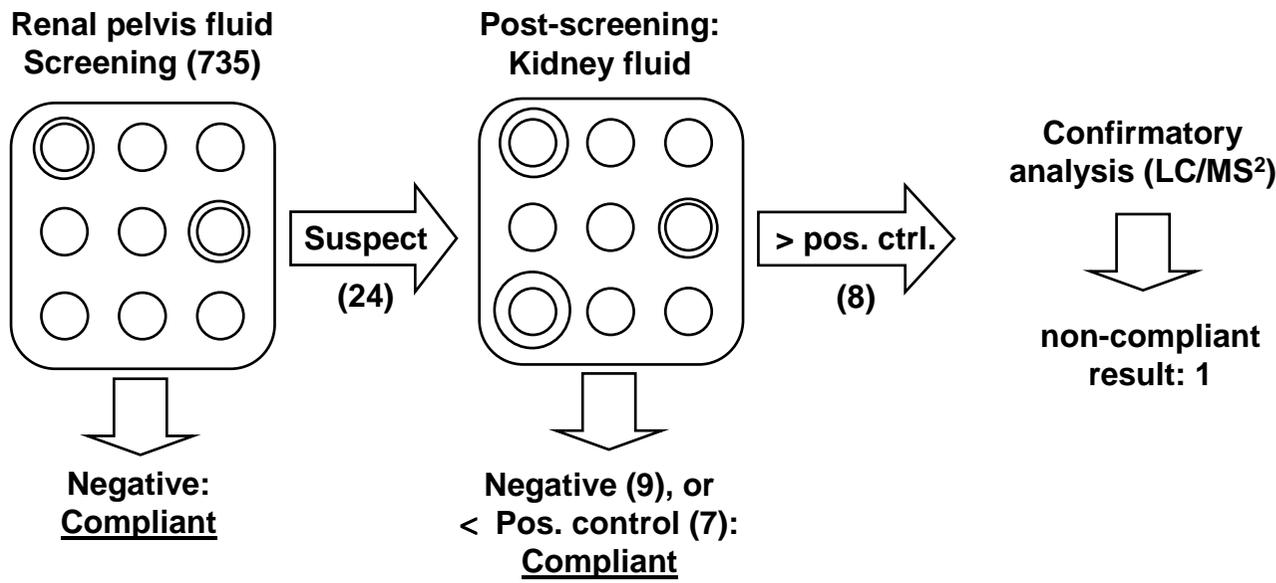
58
59
60

1
2
3 560 similar to Fig. 2a (squares: neomycin, diamonds: dihydrostreptomycin, dots: gentamicin).
4

5 561 The minimum inhibition zone equals the diameter of the punch hole, which is 14 mm.
6

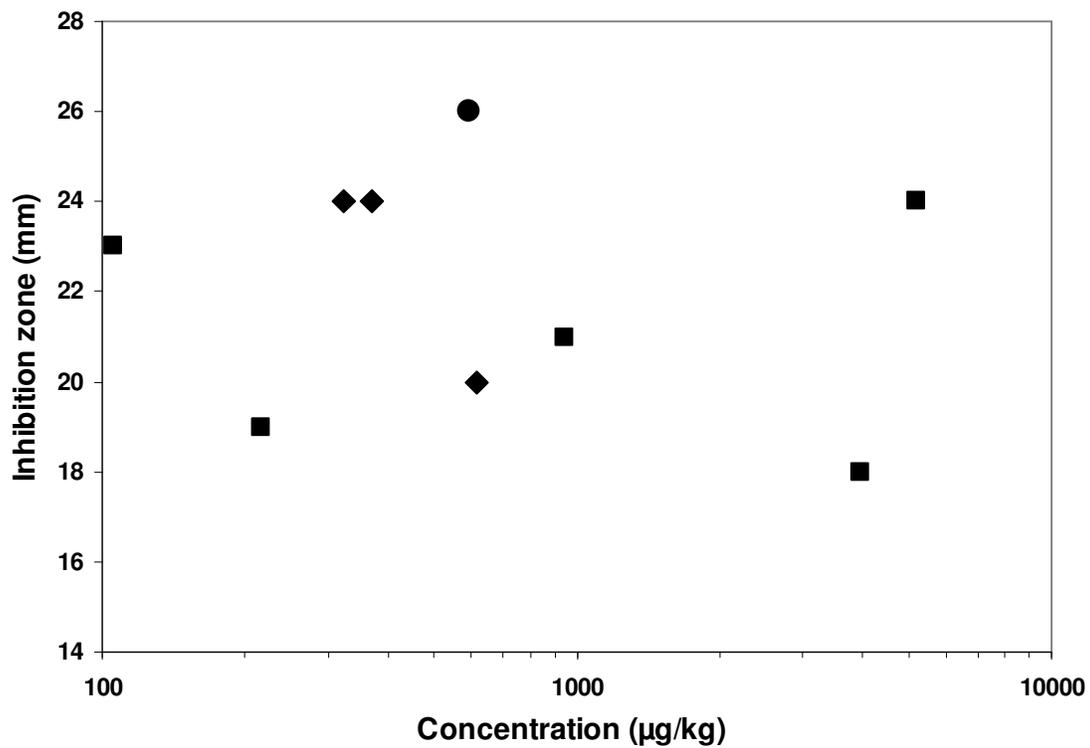
7 562
8
9
10
11
12
13
14
15
16
17
18
19
20
21
22
23
24
25
26
27
28
29
30
31
32
33
34
35
36
37
38
39
40
41
42
43
44
45
46
47
48
49
50
51
52
53
54
55
56
57
58
59
60

For Peer Review Only



1
2
3
4
5
6
7
8
9
10
11
12
13
14
15
16
17
18
19
20
21
22
23
24
25
26
27
28
29
30
31
32
33
34
35
36
37
38
39
40
41
42
43
44
45
46
47

Figure 2a.



Review Only

1
2
3
4
5
6
7
8
9
10
11
12
13
14
15
16
17
18
19
20
21
22
23
24
25
26
27
28
29
30
31
32
33
34
35
36
37
38
39
40
41
42
43
44
45
46
47
48
49
50
51
52
53
54
55
56
57
58
59
60

Figure 2b.

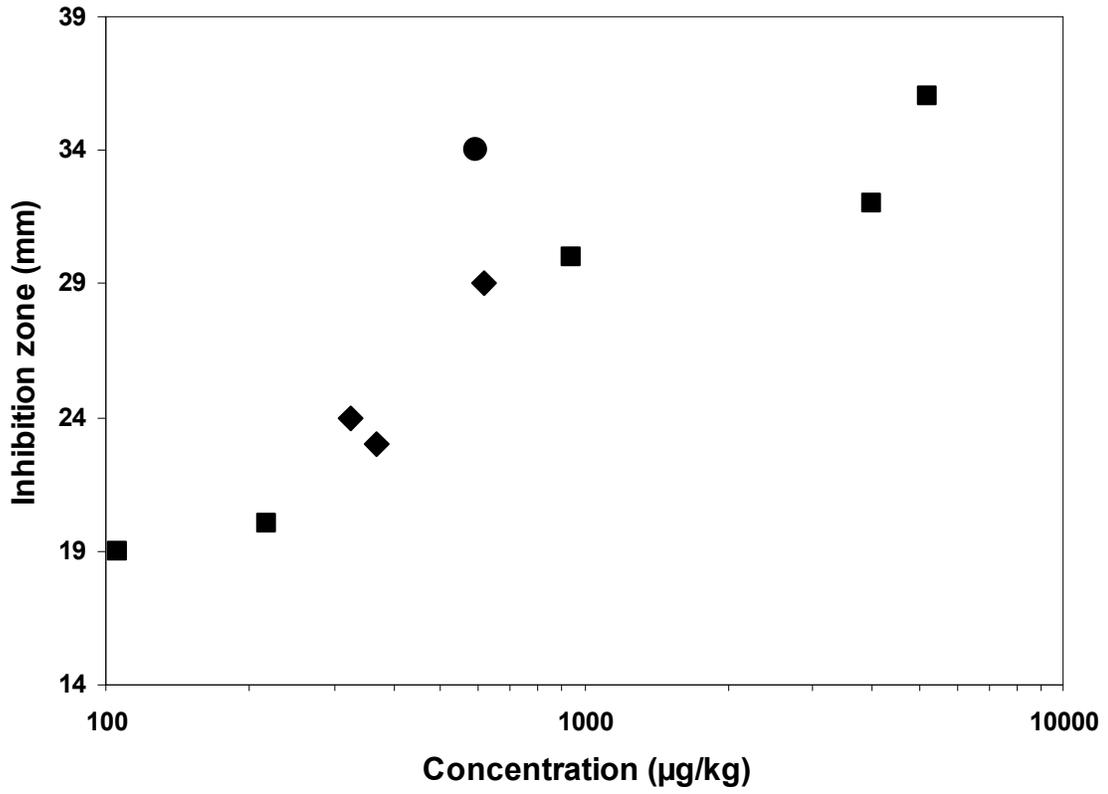


Table 1. Overview of results of individual tests on non-compliant samples encountered in this study

Non-compliant results	Matrix	MRL ($\mu\text{g kg}^{-1}$)	EU4pt	NAT	Premi [®] Test muscle	Premi [®] Test kidney
Sulfamethazine (1100 $\mu\text{g kg}^{-1}$)	muscle	100	-	+	+	+
Doxycycline (305 $\mu\text{g kg}^{-1}$)	muscle	100	+	+	-	+
Tulathromycin* (648 $\mu\text{g kg}^{-1}$)	muscle	-	+	+	-	+
Gentamicin (870 $\mu\text{g kg}^{-1}$)	kidney	750	-	-	-	+
Neomycin (5207 $\mu\text{g kg}^{-1}$)	kidney	5000	-	+	-	+

* 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 $\mu\text{g kg}^{-1}$ we consider this sample non-compliant.

Table 2. Overview of tetracycline and aminoglycoside confirmatory results on NAT suspect samples and Premi®Test positive samples

Sample	Species	NAT-screening diameter (mm)		PremiTest		NATpost-screening kidney diameter (mm)		Chemical confirmation
		tetra	amino	kidney	muscle	tetra	amino	
215	P	-	-	+	-			870 genta (k)
41	P	-	-	+	-			483 neo (k)
92	P	-	-	+	-			471 neo (k)
444	P	-	-	+	-			473 neo (k)
513	P	-	-	+	-			477 neo (k)
481	C	-	-	+	-			48 OTC/2546 neo (k)
9	C	-	-	+	-			18 OTC/23 doxy/1683 neo (k)
236	C	-	-	+	-			48 OTC/512 neo (k)
244	C	-	-	+	-			106 OTC/213 neo/147 genta (k)
266	P	31	-	+	-	35		305 doxy (m)
97	P	20	-	+	-	23		34 doxy (m)
67	P	-	24	+	-		23	369 DHS (k)
552	P	-	24	+	-		36	5207 neo (k)
651	B	-	18	+	-		32	3973 neo (k), 5 neo (m)
656	C	-	21	+	-		30	941 neo (k)
542	C	-	26	-	-		34	593 genta (k)
285	P	-	24	-	-		24	324 DHS (k)
377	P	-	20	-	-		29	614 DHS (k)
680	P	-	19	-	-		20	217 neo (k)
681	P	-	23	-	-		19	106 neo (k)
406	P	23	-	-	-	29		82 OTC (m)
696	P	25	-	-	-	27		55 OTC (m)
33	P	23	-	-	-	23		52 doxy (m)
162	P	17	-	-	-	23		42 doxy (m)
694	P	27	-	-	-	25		41 OTC (m)
329	P	17	-	-	-	19		27 doxy (m)
232	C	21	-	-	-	22		25 doxy (m)
299	P	19	-	-	-	20		25 doxy (m)
188	P	21	-	-	-	22		8 OTC, 20 doxy (m)
368	P	19	-	-	-	18		21 doxy (m)
337	C	18	-	-	-	20		17 doxy (m)
58	C	17	-	-	-	19		17 OTC/3 doxy (m)
62	C	20	-	-	-	0		7 OTC (m)
134	P	20	-	-	-	0		14 doxy (m)
386	P	18	-	-	-	0		5 doxy (m)
352	C	18	-	-	-	0		4 doxy (m)
572	P	22	-	-	-	24		20 OTC/12 doxy (m), 130 OTC/96 doxy (k)
479	P	21	-	-	-	0		26 doxy (m), 85 doxy (k)
438	P	20	-	-	-	0		7 doxy (m), 41 OTC/31 doxy (k)
667	C	28	-	-	-	0		negative (m), 167 OTC (k)
614	P	19	-	-	-	0		negative (m), 62 OTC/5 doxy (k)
575	P	19	-	-	-	0		negative (m), 32 OTC/5 doxy (k)

Abbreviations: P: porcine, C: calf, (m): muscle, (k): kidney, genta: gentamicin, neo: neomycin, DHS: dihydrostreptomycin, OTC: oxytetracycline, doxy: doxycycline.