INSTITUTE FOR AGRICULTURAL AND FISHERIES RESEARCH
TECHNOLOGY AND FOOD SCIENCE UNIT

REPORT RING TEST

"SCREENING FOR ANTIMICROBIAL SUBSTANCES WITH THE NEW BELGIAN KIDNEY TEST (NBKT)"

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1. INTRODUCTION

The Institute for Agricultural and Fisheries Research (ILVO) organised in December 2014 as National Reference Laboratory (NRL) Chemistry for substances with anabolic effect and veterinary drugs, a ring test "Screening for antimicrobial substances with the New Belgian Kidney Test (NBKT)", in collaboration with the Federal Agency for the Safety of the Food Chain (FASFC).

This proficiency test was obligatory for the following approved laboratories: CARAH, EURACETA, Eurofins Food Testing Belgium (EUROFINS-FOOD), Servaco Food Control (FOOD CONTROL), Hainaut Vigilance Sanitaire (HVS), LARECO, Laboratoire S.H.A. (LASHA), LOVAP and Quality Partner (QP).

LASHA informed ILVO and FASFC that they were not interested to participate in the ring test and hence were giving up their approval for performing the NBKT.

The labs were asked to perform the NBKT as described in the Ministerial Decree of 19 June 1995 [1] which is an amendment of the Ministerial Decree of 18 December 1973 [2].

2. PLANNING OF THE RING TEST

On November 4, 2014 the above mentioned laboratories and also some private laboratories and slaughterhouses were invited to participate. Finally, 13 laboratories subscribed to the ring test.

On December 2, a parcel containing 8 blind coded antibiotic disks (in double) was sent to the participants by postal service. The participants were asked to store the disks refrigerated (below 6°C) upon arrival and to analyse the disks in week 49.

It was asked to return the results and the interpretation of the results before the 12th of December using the specific results form.

3. SAMPLES

For this first ring test it was decided to send (spiked) antibiotic disks to the participants and no kidney material to avoid discussions about the matrix homogeneity and interferences by kidney juice.

The disks were prepared and tested at ILVO on the 1st of December. The antibiotics were chosen according to the active substances of veterinary drugs registered for pork meat in Belgium [3].

The individual codes of the antibiotic disks, each corresponding to a general sample code, are presented in Table 1.

Table 1. Codification of the antibiotic disks.

Blind coded disk spiked with	CODE												
	General	Lab 1	Lab 2	Lab 3	Lab 4	Lab 5	Lab 6	Lab 7	Lab 8	Lab 9	Lab 10	Lab 11	Lab12	Lab 13
1 µg sulfadiazine	A	7	9	22	27	33	47	49	62	65	76	88	96	104
1.5 µg sulfachloropyridazine	**B**	**6**	**15**	**19**	**26**	**39**	**43**	**55**	**60**	**69**	**79**	**82**	**90**	**98**
0.05 µg doxycycline	C	3	10	24	32	40	46	54	57	68	78	87	89	102
BLANK DISK	**D**	**1**	**16**	**21**	**29**	**36**	**44**	**51**	**63**	**71**	**73**	**83**	**91**	**103**
2 µg ceftiofur	E	8	12	20	30	35	41	56	64	70	75	86	95	97
1 µg lincomycin	**F**	**2**	**13**	**18**	**28**	**37**	**45**	**52**	**58**	**66**	**74**	**81**	**94**	**101**
1.75 µg neomycin	G	5	14	23	31	34	48	53	61	67	77	85	92	99
1 µg streptomycin	**H**	**4**	**11**	**17**	**25**	**38**	**42**	**50**	**59**	**72**	**80**	**84**	**93**	**100**

All laboratories returned the form "acknowledgement of receipt of samples" confirming that the samples arrived in good condition.

All labs received the disks on the 3rd of December except for labs 2 and 3 which received the disks on the 4th and the 5th of December, respectively.

4. SCREENING METHOD

The procedure, as described in the national legislation, recommends the use of Test Agar pH 7.2 (Merck 15/87 or equivalent), addition of 0.4% dextrose, sterilization at 121°C during 15 minutes, adjustment of the pH to 7.2 and addition of 0.2 µg of trimethoprim per ml agar and 0.1% (V/V) of a spore solution of 10^7 spores of *Bacillus subtilis* BGA per ml. A layer of 2 mm agar is obtained by pouring 14 ml agar medium in petri dishes of 9 cm diameter. Petri dishes have to be incubated at 30°C during 16-24 hours.

Since Test Agar pH 7.2 became no longer commercially available, two alternatives were suggested by Cornet *et al.* [4] namely Standard II Nutrient Agar from Merck (VWR Int.) and Niertest Agar Base from Biotrading (now commercially available at Led Techno and Tritium Microbiology, Nl). Both alternative media are slightly different in composition compared to the Test Agar pH 7.2 (Table 2). The labs are also allowed to compose their own agar medium out of single ingredients.

Table 2. Composition of test media as used by the laboratories.

Ingredient	**Quantity**		
	Test Agar pH 7.2	**Standard II Nutrient Agar**	**Niertest Agar Base**
Agar	13 g/l Bacto Agar	13 g/l Agar agar	13 g/l Agar Bacteriological No. 1
Peptone / Casitone	3.45 g/l Bacto Peptone 3.45 g/l Bacto Casitone	3.45 g/ l Peptone from meat, 3.45 g/l Peptone from casein	7 g/l Peptone bacteriological
Dextrose	/	/	4 g/l
Natriumchloride (NaCl)	5.1 g/l	5.1 g/l	5 g/l
Tri-sodiumphosphate 12 hydrate	/	/	0.8 g/l
Addition to medium			
Dextrose	4	4	/

ILVO and labs 1, 2, 8 and 11 prepared their own "Test Agar pH 7.2" medium in the right proportions starting from Bacto Agar and they added Bacto Peptone, Bacto Casitone, NaCl and dextrose. Lab 9 used petri dishes prepared by lab 8.

Labs 3, 4, 5 and 7 used Standard II Nutrient Agar and just added dextrose.

Labs 6, 10, 12 and 13 used the ready-to-use Niertest Agar Base.

All labs seeded their agar medium with the right concentration of *Bacillus subtilis* spores and added trimethoprim to their medium, however 9 out of the 13 laboratories were not adding the recommended 0.2 µg trimethoprim per ml of agar but a different concentration. Two labs were not filling the petri dishes with the recommended 14 ml of medium and two labs were incubating the plates at 37°C instead of at 30°C. Finally, one lab was not using control antibiotic disks. Table 3 gives an overview of the deviations of these labs on the national legislation.

Table 3. Overview of the deviations of the laboratories on the national legislation.

LAB	Deviations on national legislation			
	Concentration of trimethoprim Recommended: 0.2 µg/ml agar	**Volume of agar per plate Recommended: 14 ml**	**Incubation temperature Recommended: 30°C**	**Use of control antibiotic disks Recommended**
1	1 µg/ml agar	-	-	-
2	-	-	-	-
3	1 µg/ml agar	-	-	-
4	3 µg/ml agar	-	-	-
5	1 µg/ml agar	-	-	-
6	3 droplets	-	37°C	-
7	3 µg/ml agar	-	-	-
8	-	-	-	-
9	-	-		NO USE
10	1 µg/ml agar	13 ml	-	-
11	-	10 ml	-	-
12	1 µg/ml agar	-	37°C	-
13	1 µg/ml agar	-	-	-

Note: -: no deviation from the recommendation.

At ILVO some experiments were performed to check the impact of some deviations on the prescribed protocol.

An increase of the concentration of trimethoprim resulted in larger inhibition zones for sulfadiazine, sulfachloropyridazine, doxycycline, lincomycin, neomycin and streptomycin. There was no impact on the inhibition zones for disks containing ceftiofur.

Incubation at 37°C resulted in smaller inhibition zones for sulfadiazine, sulfachloropyridazine, doxycycline lincomycin, neomycin and streptomycin. Even for the quantity of lincomycin, neomycin or streptomycin used in this ring test study negative results were obtained for plates incubated at 37°C. There was no impact on the inhibition zones for disks containing ceftiofur.

Smaller layers of agar result in larger inhibition zones.

Finally, two labs (3 and 11) were not able to analyse the samples in week 49.

5. HOMOGENEITY OF THE SAMPLES

Of each sample 6 disks were ad randomly analysed at ILVO on different plates prepared with two batches of test medium and the mean diameter and the standard deviation of repeatability were calculated. The values for 1 µg sulfadiazine, 1.5 µg sulfachloropyridazine, 0.05 µg doxycycline, 2 µg ceftiofur, 1 µg lincomycin, 1.75 µg neomycin and 1 µg streptomycin were 26.9 ±2.3, 26.7 ±3.2, 28.5 ±1.7, 27.2 ±2.1, 25.8 ±2.4, 20.3 ±1.2 and 22.1 ±0.6 mm, respectively. No inhibition zones were obtained for all blank disks. For neomycin 3 out of 6 results were negative (<20 mm).

6. RESULTS AND DISCUSSION

Table 4 gives an overview of the results that the labs obtained for their control antibiotic disks.

Table 4. Inhibition zones (in mm) obtained for the control antibiotic disks.

LAB	Inhibition zone (mm)			
	Sulfadimidine 1 µg (≥17 mm)	Oxytetracycline 1 µg (≥18 mm)	Streptomycin 1 µg (≥20 mm)	Tylosin 1 µg (≥20 mm)
ILVO	23.5	29.1	23.8	30.3
1	22.8	25.0	24.1	29.1
2	23.2	32.6	25.8	31.5
3	**17.6**	23.2	24.2	24.3
4	**negative**	31	26	30
5	**17**	34	25	32
6	20.65	25.75	20.95	25.25
7	21.63	35.62	36.47	34.89
8	24.99	30.13	22.92	26.17
9	**NOT USED**	**NOT USED**	**NOT USED**	**NOT USED**
10	26.15	32.83	21.52	29.44
11	**12.70**	32.30	26.30	31.40

LAB	Inhibition zone (mm)			
	Sulfadimidine 1 µg (≥17 mm)	Oxytetracycline 1 µg (≥18 mm)	Streptomycin 1 µg (≥20 mm)	Tylosin 1 µg (≥20 mm)
12	29	32	22	30
13	25	31.5	21	27

Note: diameter of paper disk = 12.7 mm.

Lab 9 did not analyse control antibiotic disks as described in the national legislation and hence never tested the detection capabilities of their test plates.

Lab 11 did not obtain an inhibition zone for the antibiotic disk spiked with 1 µg sulfadimidine. Lab 4 also reported a negative result for the disk spiked with 1 µg sulfadimidine. Despite these negative results, the respective labs used the plates for the analysis of their samples.

Note that also the inhibition zones obtained by labs 5 and 3 are borderline and in fact too small as could be expected for a positive sulfadimidine control disk.

6.1 Disk A

Table 5. Results of disk A, spiked with 1 µg sulfadiazine.

LAB	Inhibition zone (mm)				Result according to the national legislation: positive (≥20 mm) or negative (<20 mm)
	CONTROL DISK sulfadimidine 1 µg	DISK A			
		1	2	Average	
ILVO	23.5	29.5	29.4	29.5	positive
1	22.8	33.6	32.8	33.2	positive
2	23.2	27.4	27.0	27.2	positive
3	**17.6**	**<15**	**<15**	**<15**	**negative**
4	**negative**	**/**	**15**	**15**	**negative**
5	**17**	**18**	**18**	**18**	**negative**
6	20.65	29.5	29.5	29.5	positive
7	21.63	26.11	26.06	26.09	positive
8	24.99	33.09	32.58	32.84	positive
9	**NOT USED**	**13**	**/**	**13**	**negative**
10	26.15	33.69	34.65	34.17	positive
11	**12.70**	**12.70**	**12.70**	**12.70**	**negative**
12	29	31	31	31	positive
13	25	33	33	33	positive

Lab 9 that did not use control disks reported a negative result for disk A.

The labs that reported negative results (labs 4 and 11) and the labs that reported borderline positive results (labs 5 and 3) for their control disk spiked with 1 µg sulfadimidine also reported negative results for disk A.

Remark that lab 3 reported inhibition zones <15 mm as described in the former Ministerial Decree of 18 December 1973 [2].

All other labs reported a positive result for disk A.

6.2 Disk B

Table 6. Results of disk B, spiked with 1.5 µg sulfachloropyridazine.

LAB	Inhibition zone (mm)				Result according to the national legislation: positive (≥20 mm) or negative (<20 mm)
	CONTROL DISK sulfadimidine 1 µg	DISK B			
		1	2	Average	
ILVO	23.5	30.5	29.6	30.1	positive
1	22.8	30.1	31.6	30.85	positive
2	23.2	30.05	30.3	30.2	positive
3	**17.6**	**<15**	**<15**	**<15**	**negative**
4	**negative**	**15**	**14**	**14.5**	**negative**
5	**17**	**17**	**17**	**17**	**negative**
6	20.65	30.7	32.4	31.55	positive
7	21.63	25.56	25.80	25.68	positive
8	24.99	33.42	33.92	33.67	positive
9	**NOT USED**	**13**	**/**	**13**	**negative**
10	26.15	36.27	36.93	36.60	positive
11	**12.70**	**12.70**	**12.70**	**12.70**	**negative**
12	29	32	34	33	positive
13	25	35	35	35	positive

Lab 9 that did not use control disks, reported a negative result for disk B.

The labs that reported negative results (labs 4 and 11) and the labs that reported borderline positive results (labs 5 and 3) for their control disk spiked with 1 µg sulfadimidine also reported negative results for disk B.

Remark that lab 3 reported inhibition zones <15 mm as described in the former Ministerial Decree of 18 December 1973 [2].

All other labs reported a positive result for disk B.

6.3 Disk C

Table 7. Results of disk C, spiked with 0.05 µg doxycycline.

LAB	Inhibition zone (mm)				Result according to the national legislation: positive (≥20 mm) or negative (<20 mm)
	CONTROL DISK Oxytetracycline 1 µg	DISK C			
		1	2	Average	
ILVO	29.1	30.4	30.6	30.5	positive
1	25.0	29.2	28.8	29	positive
2	32.6	30.5	29.8	30.15	positive
3	23.2	22.9	22.7	22.8	positive
4	31	30	30	30	positive
5	34	29	29	29	positive
6	25.75	26.6	26.2	26.4	positive
7	**35.62**	**35.07**	**34.73**	**34.90**	positive
8	30.13	29.27	28.91	29.09	positive
9	**NOT USED**	**15**	**/**	**15**	**negative**
10	32.83	29.79	29.11	29.45	positive
11	32.30	32.70	31.00	31.90	positive
12	32	29	26	27.5	positive
13	31.5	28	28	28	positive

Only lab 9 reported a negative result for disk C; this lab did not use control disks.

All other labs reported a positive result for disk C.

Remark the large inhibition zones obtained by lab 7, probably caused by the high concentration of trimethoprim (addition of 3 µg trimethoprim per ml of agar).

6.4 Disk D

Table 8. Results of disk D, a blank disk.

LAB	Inhibition zone (mm)			Result according to the national legislation: positive (≥20 mm) or negative (<20 mm)
	DISK D			
	1	2	Average	
ILVO	12.7	12.7	12.7	negative
1	13	13	13	negative
2	<13	<13	<13	negative
3	**<15**	**<15**	**<15**	negative
4	**/**	**/**	**/**	negative
5	12	12	12	negative
6	12.7	12.7	12.7	negative
7	<13	<13	<13	negative
8	13	13	13	negative
9	13	/	13	negative
10	**<1**	**<1**	**<1**	negative
11	12.70	12.70	12.70	negative
12	**no**	**no**	**no**	negative
13	13	13	13	negative

Disk D was a blank disk free from antimicrobial substances.

All laboratories found a negative result for this blank disk. Hence, no false positive results were obtained.

The national legislation asks to measure the diameter of the inhibition zones, including the paper disks (with a diameter of 12.7 mm). Note that labs 4, 10 and 12 did not take into account the diameter of their paper disks in their result.

Remark that lab 3 reported inhibition zones <15 mm as described in the former Ministerial Decree of 18 December 1973 [2].

6.5 Disk E

Table 9. Results of disk E, spiked with 2 µg ceftiofur.

LAB	Inhibition zone (mm)			Result according to the national legislation: positive (≥20 mm) or negative (<20 mm)
	DISK E			
	1	2	Average	
ILVO	24.1	25.0	24.6	positive
1	24.4	26.6	25.5	positive
2	20.9	21.4	21.15	positive
3	25.7	25.9	25.8	positive
4	**17**	**17**	**17**	**negative**
5	24	22	23	positive
6	**45.4**	**44.5**	**44.95**	positive
7	25.66	26.30	25.98	positive
8	23.02	22.50	22.76	positive
9	24	/	24	positive
10	33.17	34.25	33.71	positive
11	**42.10**	**46.10**	**44.10**	positive
12	29	28	28.5	positive
13	**48**	**50**	**49**	positive

With the exception of lab 4 that reported a negative result for disk E, the other labs reported a positive result for disk E.

It is worth noting that some labs (6, 11 and 13) reported extremely large inhibition zones. Remark that lab 11 poured smaller layers of agar in the petri dishes which results in larger inhibition zones.

6.6 Disk F

Table 10. Results of disk F, spiked with 1 µg lincomycin.

LAB	Inhibition zone (mm)			Result according to the national legislation: positive (≥20 mm) or negative (<20 mm)
	DISK F			
	1	2	Average	
ILVO	29.7	27.2	28.5	positive
1	24.7	23.5	24.1	positive
2	26.6	26.7	26.65	positive
3	**<15**	**<15**	**<15**	**negative**
4	26	26	26	positive
5	20	21	21	positive
6	**16.1**	**16.0**	**16.05**	**negative**
7	**30.17**	**31.05**	**30.61**	positive
8	**18.51**	**19.07**	**18.79**	**negative**
9	**13**	**/**	**13**	**negative**
10	**19.36**	**19.34**	**19.35**	**negative**
11	**31.00**	**29.00**	**30.00**	positive
12	**19**	**18**	**18.5**	**negative**
13	21	21	21	positive

Lab 9 that did not use control disks, reported a negative result for disk F.

Labs 3, 6, 8, 10 and 12 reported a negative result for disk F.

Remark the larger inhibition zones obtained by lab 11, probably due to the use of plates containing only 10 ml of agar and by lab 7, probably caused by the addition of 3 µg trimethoprim per ml of agar.

Since lab 3 was not able to analyse the samples in week 49 due to a late postal delivery, a simulation was carried out at ILVO demonstrating that a delay in the analysis had no impact on the test result of disk F.

6.7 Disk G

Table 11. Results of disk G, spiked with 1.75 µg neomycin.

LAB	Inhibition zone (mm)			Result according to the national legislation: positive (≥20 mm) or negative (<20 mm)
	DISK G			
	1	2	Average	
ILVO	20.9	21.2	21.1	positive
1	**18.2**	**19.5**	**18.85**	**negative**
2	24.0	23.0	23.5	positive
3	22.5	22.7	22.6	positive
4	**24**	**25**	**24.5**	positive
5	20	22	21	positive
6	**18.1**	**18.6**	**18.35**	**negative**
7	**25.70**	**24.55**	**25.13**	positive
8	**18.11**	**18.33**	**18.22**	**negative**
9	**15**	**/**	**15**	**negative**
10	**18.57**	**18.96**	**18.77**	**negative**
11	**24.80**	**23.40**	**24.10**	positive
12	**20**	**19**	**19.5**	**negative**
13	21	21	21	positive

Lab 9 that did not use control disks reported a negative result for disk G.

Also labs 1, 6, 8, 10 and 12 reported a negative result for disk G. Negative results could be expected; in the homogeneity testing at ILVO also negative results were obtained for this disk.

Remark the slightly larger inhibition zones obtained by lab 11, probably due to the use of plates containing only 10 ml of agar and by labs 4 and 7, probably caused by the addition of 3 µg trimethoprim per ml of agar.

6.8 Disk H

Table 12. Results of disk H, spiked with 1 µg streptomycin.

LAB	Inhibition zone (mm)				Result according to the national legislation: positive (≥20 mm) or negative (<20 mm)
	CONTROL DISK Streptomycin 1 µg	DISK H			
		1	2	Average	
ILVO	23.8	23.0	22.8	22.9	positive
1	24.1	25	24.9	24.95	positive
2	25.8	24.0	24.4	24.2	positive
3	24.2	23.9	24.1	24.0	positive
4	**26**	**27**	**27**	**27**	positive
5	25	25	24	25	positive
6	20.95	20.4	20.6	20.5	positive
7	**36.47**	**30.37**	**30.37**	**30.37**	positive
8	22.92	22.31	22.55	22.43	positive
9	**NOT USED**	**15**	**/**	**15**	**negative**
10	21.52	21.96	21.97	21.97	positive
11	**26.30**	**28.00**	**26.40**	**27.20**	positive
12	22	21	21	21	positive
13	21	22	22	22	positive

Lab 9 that did not use control disks reported a negative result for disk H.

All other labs reported a positive result for disk H.

Remark the larger inhibition zones obtained by lab 7 and to a lesser extent by lab 4, probably caused by the addition of 3 µg trimethoprim per ml of agar and by lab 11, probably due to the use of plates containing only 10 ml of agar.

7. CONCLUSIONS

Table 13 gives an overview of the results obtained by the laboratories.

Table 13. Overview of the results per lab and per sample (13 labs, 8 samples)

LAB	Number of correct results*	Number of false positive results	Number of false negative results
1	8	0	0
2	8	0	0
3	5	0	3
4	5	0	3
5	6	0	2
6	7	0	1
7	8	0	0
8	7	0	1
9	3	0	5
10	7	0	1
11	6	0	2
12	7	0	1
13	8	0	0

Note: * both negative and positive results for disk G (1.75 µg neomycin) were considered as correct.

DISK	Compound	Number of correct results*
A	sulfadiazine	8
B	sulfachloropyridazine	8
C	doxycycline	12
D	-	13
E	ceftiofur	12
F	lincomycin	7
G	neomycin	13
H	streptomycin	12

No false positive results were obtained.

No false negative results were obtained by labs 2, 7 and 13. Taking into account that disk G, spiked with 1.75 µg of neomycin, was also missed in some cases in the homogeneity study at ILVO, it can be concluded that no false negative results were obtained by lab 1.

A large variation is observed in the results. In reality even a larger variation can be expected since in this ring trial no manipulation in the handling of kidneys is included. It is clear that not all laboratories monitor pork carcasses at the same antimicrobial residue level. Several false negative results were obtained; none of the doped filter disks was tested as positive by all laboratories: 1 µg sulfadiazine, 1.5 µg sulfachloropyridazine, 0.05 µg doxycycline, 2 µg ceftiofur, 1 µg lincomycin, 1.75 µg neomycin and 1 µg streptomycin were considered as negative by 5, 5, 1, 1, 6, 6 and 1

out of 13 laboratories, respectively. Especially the detection of residues of sulfa drugs is problematic in some laboratories (labs 3, 4, 5, 9 and 11) despite sulfonamides are the most occurring residues in pork meat (FASFC, results national residue plan). Some of these problems are related to the fact that the legislative protocol is not strictly followed. Some labs even use test plates despite the fact that the control disks are indicating problems with the detection capability of the plates. Part of the problem could be prevented by the use of control disks and refusal of the plates with control disks not meeting the criteria.

It is worth noting that laboratory 9 is not only not using control disks, but is also not performing the test *in duplo* as requested in the legislative protocol [1].

Laboratories should strictly follow the prescribed protocol (test medium, added concentration of trimethoprim, volume of agar in petri dish, incubation temperature, duplicate analyses, …). This also implies the compulsory use of control disks, and subsequently, the disqualification of plates not fulfilling the criteria (i.e. plates shown not to be fit for purpose, are not be used).

8. REFERENCES

[1] Ministerieel Besluit van 19 juni 1995 tot bepaling van de laboratoriumtechnieken voor het opsporen van residuen van stoffen met een kiemgroeiremmende werking (Ministerial Decree of 19 June 1995).

[2] Ministerieel Besluit van 18 december 1973 tot bepaling van de laboratoriumtechnieken voor het opsporen van residuen van stoffen met een kiemgroeiremmende werking (Ministerial Decree of 18 December 1973).

[3] *Anonymous* (2014). Gecommentarieerd geneesmiddelenrepertorium voor diergeneeskundig gebruik. Belgisch Centrum voor Farmacotherapeutische Informatie 2014, Brussel: 1-326.

[4] Cornet V., Govaert Y., Koenen-Dierick K., Okerman L. and Degroodt J.M. (2005). Interlaboratory study based on a one-plate screening method for the detection of antibiotic residues in bovine kidney tissue. Food Additives and Contaminants, 22 (5): 415-422.