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)"

12 February 2016

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1. INTRODUCTION

The Institute for Agricultural and Fisheries Research (ILVO) organised in November 2015 as National Reference Laboratory (NRL) Chemistry for substances with anabolic effect and veterinary drugs - in consortium with CER-Groupe - a second ring test "Screening for antimicrobial substances with the New Belgian Kidney Test" in collaboration with the Federal Agency for the Safety of the Food Chain (FASFC). This ring test was organised to follow up the unsatisfactory results that some laboratories obtained for the previous ring test [1].

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, LOVAP and Quality Partner (QP).

The labs were asked to perform the NBKT as described in the Ministerial Decree of 19 June 1995 [2] which is an amendment of the Ministerial Decree of 18 December 1973 [3].

2. PLANNING OF THE RING TEST

On 23 October 2015, the above mentioned laboratories and also some private laboratories and slaughterhouses were invited to participate. Finally, 14 laboratories subscribed to the ring test. In order to facilitate the comparison of the results of the labs for both ring tests, the anonymous codes of the labs were retained.

On 24 November, 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 48.

It was asked to return the results and the interpretation of the results before 4 December using the specific results form.

3. SAMPLES

Since the results of the first ring test were not very satisfactory, it was decided to send again (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 24 November. The antibiotics were chosen according to the active substances of veterinary drugs registered for pork in Belgium [4].

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	Lab 14
1 µg neomycin	A	6	10	23	27	38	47	54	59	68	74	86	89	103	107
/ (blank disk)	B	1	11	17	26	36	43	55	60	65	79	88	93	101	105
0.5 µg tylosin	C	4	16	22	31	37	46	49	63	67	73	82	90	97	108
1 µg sulfadiazine	D	2	12	20	29	40	44	52	57	69	78	85	96	104	110
0.25 µg tylosin	E	3	9	18	25	33	45	56	61	72	75	87	91	100	109
1.5 µg sulfachloropyridazine	F	5	13	24	28	34	42	50	64	71	80	81	92	99	106
2 µg ceftiofur	G	8	15	19	30	35	48	51	62	66	76	84	94	98	112
0.05 µg doxycycline	H	7	14	21	32	39	41	53	58	70	77	83	95	102	111

Laboratories 4, 9, 10 and 14 did not return the form "acknowledgement of receipt of samples" confirming that the samples arrived in good condition.

All labs received the disks on 25 November, except for labs 12 and 9. Lab 12 received the disks on 26 November; lab 9 didn't receive the antibiotic disks in week 48. So, another set of disks was sent on 1 December to lab 9, addressed to the indicated contact person. Unfortunately, these disks also didn't arrive at lab 9; they were automatically forwarded to a partner lab (lab 8) where they only arrived on 7 December.

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.* [5] namely Standard II Nutrient Agar from Merck (commercially available at VWR Int.) and Niertest Agar (Base) from Biotrading (Tritium Microbiologie (NL), commercially available at Led Techno and Bio-Rad Laboratories). The medium of Tritium Microbiologie is available in ready-to-use plates (N001) and in bottles to which trimethoprim and spores have to be added (N003). Recently, the composition of the ready-to-use plates has been changed by Tritium Microbiologie (available as N022).

These alternative media are slightly different in composition compared to the Test Agar pH 7.2.

The labs are also allowed to compose their own agar medium out of single ingredients.

ILVO and labs 1, 2 and 8 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 5 and 7 used Standard II Nutrient Agar and added dextrose.

Labs 6, 10, 12 and 13 used the Niertest Agar Base of Tritium Microbiologie (N003); labs 3, 11 and 14 used the "improved" ready-to-use Niertest Agar plates of Tritium Microbiologie (N022).

Lab 4 analysed one disk (disk 1) with their own prepared medium and one disk (disk 2) with Niertest Agar Base (N003). Since no opposite results were obtained using these two media, their results were processed together.

All labs that didn't use ready-to-use plates seeded their agar medium with the right concentration of *Bacillus subtilis* spores and added trimethoprim to their medium, however in different concentrations ranging from the recommended 0.2 µg trimethoprim per ml of agar till 3 µg trimethoprim per ml of agar. It is known that an increase of the concentration of trimethoprim is resulting in larger inhibition zones, especially for sulphonamides.

Lab 12 filled the petri dishes with 15 ml of medium instead of the recommended 14 ml and also incubated the plates at 37°C instead of at the recommended 30°C. It is known that bigger layers of agar and incubation at 37°C result in smaller inhibition zones (for the most antibiotics).

Laboratory 10 was not performing the test *in duplo* as requested in the legislative protocol [2].

Lab 14 was not using a control antibiotic disk for the group of sulphonamides and did not interpret the results according to the legislative protocol [2].

Finally, five labs (4, 5, 9, 10 and 12) did not analyse the samples in week 48.

Lab 5 first analysed the antibiotic disks with Niertest Agar of Tritium Microbiologie (N003) but did not obtain sufficient inhibition for the control antibiotic disks of sulfadimine and streptomycin. So, a new set of disks was given to lab 5 on 7 December.

Due to the late arrival of the disks and other deadlines in week 49, lab 12 only analysed the disks on 11 December.

On request of ILVO it was decided that lab 9 (and lab 8 each) analysed one disk of the second shipment in order to be able to evaluate lab 9. Hence, lab 9 is excused for analysing only one disk in this ring trial. Finally, both labs analysed the disks on 10 or 11 December.

5. HOMOGENEITY OF THE SAMPLES

Of each sample 6 disks were randomly and in double analysed at ILVO on different plates and the mean diameter and the standard deviation were calculated. The values for 1 µg neomycin, 0.5 µg tylosin, 1 µg sulfadiazine, 0.25 µg tylosin, 1.5 µg sulfachloropyridazine, 2 µg ceftiofur and 0.05 µg doxycycline were 18.0±0.4, 25.8±0.2, 27.2±0.6, 21.1±0.6, 25.8±0.3, 25.0±0.6 and 26.9±0.7 mm, respectively. No inhibition zones were obtained for all blank disks.

6. RESULTS AND DISCUSSION

In order to facilitate the comparison of the results of both ring tests, the results of the previous ring test are mentioned in between brackets after the current results (if performed). Remark that lab 14 did not participate in the previous ring test.

Table 2 gives an overview of the results that the labs obtained for their control antibiotic disks.

Table 2. 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	23.9 (23.5)	27.7 (29.1)	23.2 (23.8)	29.3 (30.3)
1	19.81 (22.8)	30.73 (25.0)	15.91 (24.1)	26.66 (29.1)
2	20.4 (23.2)	28,5 (32.6)	22.2 (25.8)	27.7 (31.5)
3	20.4 (17.6)	30.3 (23.2)	20.1 (24.2)	24.0 (24.3)
4	20.5 (negative)	27.5 (31)	22 (26)	30 (30)
5	19.6 (17)	36.0 (34)	30.5 (25)	33.4 (32)
6	20.9 (20.65)	26.2 (25.75)	20.4 (20.95)	23.4 (25.25)
7	20.0 (21.63)	30.3 (35.62)	25.5 (36.47)	29.8 (34.89)
8	22.0 (24.99)	25.2 (30.13)	26.0 (22.92)	30.5 (26.17)
9	21.7 (not used)	21.6 (not used)	25.4 (not used)	31.2 (not used)
10	21.13 (26.15)	29.18 (32.83)	20.69 (21.52)	26.34 (29.44)
11	16.60 (12.70)	32.90 (32.30)	18.20 (26.30)	24.70 (31.40)
12	28 (29)	34 (32)	25 (22)	32 (30)
13	18 (25)	21 (31.5)	25 (21)	29 (27)
14	NOT USED	19.0	negative	24.5

Note: diameter of paper disk = 12.7 mm; results of previous ring test in between brackets.

Lab 14 did not use a sulfadimidine control disk as described in the national legislation and hence has no idea about the sensitivity of their test plates for the group of the sulphonamides. Lab 14 also reported a negative result for the disk spiked with 1 µg streptomycin.

Also the inhibition zones obtained by lab 11 for the sulfadimidine and the streptomycin control disks are too small.

Remark that labs 11 and 14 – but also lab 3 - were using the ready-to-use Niertest Agar plates (N022) of Tritium Microbiologie.

Finally, the inhibition zone obtained by lab 1 for the streptomycin control disk is insufficient.

In fact, when the control disks are not meeting the criteria, the test plates should be **refused**.

6.1 Disk A

Table 3. Results of disk A, spiked with 1 µg neomycin.

LAB	Inhibition zone for DISK A (mm)			Result according to the national legislation: positive (≥20 mm) or negative (<20 mm)
	1	2	Average	
ILVO	18.1	17.9	17.9	negative
1	16.19	16.19	16.19	negative
2	18.4	18.8	18.6	negative
3	<15	<15	<15	negative
4	19	18	18.5	negative
5	21.6	21.7	21.7	positive
6	13.2	14.9	14.1	negative
7	22.0	22.7	22.4	positive
8	20.1	20.6	20.4	positive
9	19.4	/	19.4	negative
10	15.21	/	15.21	negative
11	18.70	17.10	17.90	positive
12	18	18.5	18.25	positive
13	20	20	20	positive
14	negative	negative	negative	negative

Note: diameter of paper disk = 12.7 mm.

Disk A should give inhibition zones between 12.7 and 19.9 mm and hence a negative result but inhibition zones (slightly) bigger than 20 mm (and hence a positive result) are unavoidable.

Labs 3 and 14 reported no inhibition zones for disk A which is not possible. Hence, labs 3 and 14 obtained **false negative results**. Note that these labs – but also lab 11 - were using the ready-to-use Niertest Agar plates (N022) of Tritium Microbiologie.

Labs 11 and 12 reported inhibition zones smaller than 20 mm for disk A but didn't interpret their result according to the national legislation [2]. Lab 11 declared to have reported on purpose a positive result since two of their control disks were not fulfilling the criteria. Remark that labs 11 and 14 also obtained negative results for the streptomycin control disk, another compound of the group of the aminoglycosides.

6.2 Disk B

Table 4. Results of disk B, a blank disk.

LAB	Inhibition zone for DISK B (mm)			Result according to the national legislation: positive (≥20 mm) or negative (<20 mm)
	1	2	Average	
ILVO	12.7 (12.7)	12.7 (12.7)	12.7 (12.7)	negative (negative)
1	13 (13)	13 (13)	13 (13)	negative (negative)
2	13 (<13)	13 (<13)	13 (<13)	negative (negative)
3	<15 (<15)	<15 (<15)	<15 (<15)	negative (negative)
4	12 (/)	12 (/)	12 (/)	negative (negative)
5	12.7 (12)	12.7 (12)	12.7 (12)	negative (negative)
6	12.7 (12.7)	12.7 (12.7)	12.7 (12.7)	negative (negative)
7	<13 (<13)	<13 (<13)	<13 (<13)	negative (negative)
8	13 (13)	13 (13)	13 (13)	negative (negative)
9	13 (13)	/ (/)	13 (13)	negative (negative)
10	0 (<1)	/ (<1)	0 (<1)	negative (negative)
11	12.90 (12.70)	12.90 (12.70)	12.90 (12.70)	negative (negative)
12	/ (no)	/ (no)	/ (no)	negative (negative)
13	13 (13)	13 (13)	13 (13)	negative (negative)
14	negative	negative	negative	negative

Note: diameter of paper disk = 12.7 mm; results of previous ring test in between brackets.

Disk B 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 [2] imposes to measure the diameter of the inhibition zones, including the paper disks (with a diameter of 12.7 mm). Note that labs 10, 12 and 14 did not take into account the diameter of their paper disks in their result.

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

6.3 Disk C

Table 5. Results of disk C, spiked with 0,5 µg tylosin.

LAB	Inhibition zone (mm)				Result according to the national legislation: positive (≥20 mm) or negative (<20 mm)
	CONTROL DISK Tylosin 1 µg	DISK C			
		1	2	Average	
ILVO	29.3	25.8	26.0	25.9	positive
1	26.66	23.91	23.55	23.73	positive
2	27.7	25.2	25.5	25.4	positive
3	24.0	20.6	21.0	20.8	positive
4	30	26	25	25.5	positive
5	33.4	31.0	31.2	31.1	positive
6	23.4	21.1	21.5	21.3	positive
7	29.8	29.5	30.5	30.0	positive
8	30.5	27.9	26.5	27.2	positive
9	31.2	27.5	/	27.5	positive
10	26.34	24.51	/	24.51	positive
11	24.70	21.50	20.70	21.10	positive
12	32	27.5	27.5	27.5	positive
13	29	26	27	26.5	positive
14	24.5	16.5	16.4	16.5	~~positive~~

Note: diameter of paper disk = 12.7 mm.

All labs reported a positive result for disk C.

However, lab 14 didn't interpret the result according to the national legislation [2]: they reported a positive result for an average inhibition zone of 16.5 mm (< 20 mm). Hence, lab 14 obtained in fact a **false negative result**.

6.4 Disk D

Table 6. Results of disk D, 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 D			
		1	2	Average	
ILVO	23.9 (23.5)	30.0 (29.5)	30.9 (29.4)	30.5 (29.5)	positive (positive)
1	19.81 (22.8)	28.95 (33.6)	28.25 (32.8)	28.6 (33.2)	positive (positive)
2	20.4 (23.2)	26.6 (27.4)	26.0 (27.0)	26.3 (27.2)	positive (positive)
3	20.4 (17.6)	29.0 **(<15)**	28.6 **(<15)**	28.8 **(<15)**	positive **(negative)**
4	20.5 **(negative)**	23 **(/)**	25 **(15)**	24 **(15)**	positive **(negative)**
5	19.6 (17)	25.3 **(18)**	25.7 **(18)**	25.5 **(18)**	positive **(negative)**
6	20.9 (20.65)	26.1 (29.5)	29.0 (29.5)	27.6 (29.5)	positive (positive)
7	20.0 (21.63)	26.8 (26.11)	27.7 (26.06)	27.2 (26.09)	positive (positive)
8	22.0 (24.99)	25.1 (33.09)	26.3 (32.58)	25.7 (32.84)	positive (positive)
9	21.7 **(not used)**	27.9 **(13)**	/ **(/)**	/ **(13)**	positive **(negative)**
10	21.13 (26.15)	26.26 (33.69)	/ (34.65)	26.26 (34.17)	positive (positive)
11	16.60 **(12.70)**	27.65 **(12.70)**	26.50 **(12.70)**	27.08 **(12.70)**	positive **(negative)**
12	28 (29)	34.5 (31)	34 (31)	34.25 (31)	positive (positive)
13	18 (25)	21 (33)	21 (33)	21 (33)	positive (positive)
14	NOT USED	17.2	17.1	17.2	~~positive~~

Note: diameter of paper disk = 12.7 mm; results of previous ring test in between brackets.

All labs reported a positive result for disk D.

However, lab 14 didn't interpret the result according to the national legislation [2]: they reported a positive result for an average inhibition zone of 17.2 mm (<20 mm). Hence, lab 14 obtained in fact a **false negative result**. Note that the same lab 14 did not use a sulfadimidine control disk.

Remark that lab 11 that obtained a borderline negative result for the sulfadimidine control disk obtained a positive result for disk D.

6.5 Disk E

Table 7. Results of disk E, spiked with 0.25 µg tylosin.

LAB	Inhibition zone (mm)				Result according to the national legislation: positive (≥20 mm) or negative (<20 mm)
	CONTROL DISK Tylosin 1 µg	DISK E			
		1	2	Average	
ILVO	29.3	22.9	22.2	22.6	positive
1	26.66	20.2	17.24	18.72	negative
2	27.7	21.3	21.0	21.2	positive
3	24.0	19.0	19.8	19.4	negative
4	30	22	20	21	positive
5	33.4	26.4	26.4	26.4	positive
6	23.4	16.5	18.7	17.6	negative
7	29.8	27.1	27.6	27.3	positive
8	30.5	22.9	23.1	23.0	positive
9	31.2	22.1	/	22.1	positive
10	26.34	19.99	/	19.99	negative
11	24.70	15.50	17.00	16.25	negative
12	32	24	22.5	23.25	positive
13	29	23	23	23	positive
14	24.5	14.0	13.8	13.9	~~positive~~

Note: diameter of paper disk = 12.7 mm.

Labs 2, 4, 5, 7, 8, 9, 12 and 13 reported a positive result for disk E; labs 1, 3, 6, 10 and 11 obtained a negative result.

So, **false negative results** are obtained by labs 1, 3, 6, 10 and 11.

Lab 14 didn't interpret the result according to the national legislation [2]: they reported a positive result for an average inhibition zone of 13.9 mm (<20 mm). Hence, lab 14 obtained in fact a **false negative result**.

Remark that the labs that obtained the smallest inhibition zones for the tylosin control disk obtained a negative result for disk E.

6.6 Disk F

Table 8. Results of disk F, 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 F			
		1	2	Average	
ILVO	23.9 (23.5)	28.4 (30.5)	29.5 (29.6)	29.0 (30.1)	positive (positive)
1	19.81 (22.8)	25.02 (30.1)	26.04 (31.6)	25.53 (30.85)	positive (positive)
2	20.4 (23.2)	25.2 (30.05)	25.7 (30.3)	25.4 (30.2)	positive (positive)
3	20.4 (17.6)	27.3 **(<15)**	27.0 **(<15)**	27.2 **(<15)**	positive **(negative)**
4	20.5 **(negative)**	21 **(15)**	25 **(14)**	23 **(14.5)**	positive **(negative)**
5	19.6 (17)	25.4 **(17)**	25.5 **(17)**	25.5 **(17)**	positive **(negative)**
6	20.9 (20.65)	28.2 (30.7)	30.3 (32.4)	29.3 (31.55)	positive (positive)
7	20.0 (21.63)	24.3 (25.56)	24.8 (25.80)	24.6 (25.68)	positive (positive)
8	22.0 (24.99)	23.5 (33.42)	27.0 (33.92)	25.3 (33.67)	positive (positive)
9	21.7 **(not used)**	27.1 **(13)**	/ **(/)**	27.1 **(13)**	positive **(negative)**
10	21.13 (26.15)	29.58 (36.27)	/ (36.93)	29.58 (36.60)	positive (positive)
11	16.60 **(12.70)**	24.20 **(12.70)**	23.40 **(12.70)**	23.80 **(12.70)**	positive **(negative)**
12	28 (29)	36.5 (32)	36.5 (34)	36.5 (33)	positive (positive)
13	18 (25)	22 (35)	22 (35)	22 (35)	positive (positive)
14	NOT USED	19.7	19.4	19.6	~~positive~~

Note: diameter of paper disk = 12.7 mm; results of previous ring test in between brackets.

All labs reported a positive result for disk F.

However, lab 14 didn't interpret the result according to the national legislation [2]: they reported a positive result for an average inhibition zone of 19.6 mm (<20 mm). Hence, lab 14 obtained in fact a **false negative result**. Note that the same lab 14 did not use a sulfadimidine control disk.

Remark that lab 11 that obtained a borderline negative result for the sulfadimidine control disk obtained a positive result for disk F.

6.7 Disk G

Table 9. Results of disk G spiked with 2 µg ceftiofur.

LAB	Inhibition zone for DISK G (mm)			Result according to the national legislation: positive (≥20 mm) or negative (<20 mm)
	1	2	Average	
ILVO	26.7 (24.1)	25.7 (25.0)	26.2 (24.6)	positive (positive)
1	26.2 (24.4)	25.63 (26.6)	25.81 (25.5)	positive (positive)
2	17.4 (20.9)	18.3 (21.4)	17.8 (21.15)	negative (positive)
3	28.4 (25.7)	27.8 (25.9)	28.1 (25.8)	positive (positive)
4	29 (17)	42 (17)	35.5 (17)	positive (negative)
5	27.9 (24)	28.2 (22)	28.1 (23)	positive (positive)
6	20.3 (45.4)	20.9 (44.5)	20.6 (44.95)	positive (positive)
7	20.1 (25.66)	20.4 (26.30)	20.2 (25.98)	positive (positive)
8	26.4 (23.02)	24.9 (22.50)	25.7 (22.76)	positive (positive)
9	25.7 (24)	/ (/)	25.7 (24)	positive (positive)
10	0 (33.17)	/ (34.25)	0 (33.71)	negative (positive)
11	32.20 (42.10)	31.00 (46.10)	31.60 (44.10)	positive (positive)
12	/ (29)	/ (28)	/ (28.5)	negative (positive)
13	43 (48)	43 (50)	43 (49)	positive (positive)
14	40.0	44.1	42.1	positive

Note: diameter of paper disk = 12.7 mm; results of previous ring test in between brackets.

Labs 2, 10 and 12 reported a negative result for disk G; the other labs reported a positive result for disk G.

Hence, labs 2, 10 and 12 obtained **false negative results**.

Note that labs 10 and 12 reported no inhibition zones at all for disk G !

6.8 Disk H

Table 10. Results of disk H, 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 H			
		1	2	Average	
ILVO	27.7 (29.1)	28.0 (30.4)	29.3 (30.6)	28.6 (30.5)	positive (positive)
1	30.73 (25.0)	27.32 (29.2)	27.2 (28.8)	27.26 (29)	positive (positive)
2	28.5 (32.6)	27.5 (30.5)	27.2 (29.8)	27.4 (30.15)	positive (positive)
3	30.3 (23.2)	28.4 (22.9)	27.9 (22.7)	28.2 (22.8)	positive (positive)
4	27.5 (31)	25 (30)	29 (30)	27 (30)	positive (positive)
5	36.0 (34)	33.6 (29)	33.7 (29)	33.7 (29)	positive (positive)
6	26.2 (25.75)	25.4 (26.6)	25.8 (26.2)	25.6 (26.4)	positive (positive)
7	30.3 (35.62)	30.1 (35.07)	30.6 (34.73)	30.3 (34.90)	positive (positive)
8	25.2 (30.13)	25.1 (29.27)	25.6 (28.91)	25.4 (29.09)	positive (positive)
9	21.6 **(not used)**	24.1 **(15)**	/ **(/)**	24.1 **(15)**	positive **(negative)**
10	29.18 (32.83)	24.45 (29.79)	/ (29.11)	24.45 (29.45)	positive (positive)
11	32.90 (32.30)	28.30 (32.70)	27.35 (31.00)	27.83 (31.90)	positive (positive)
12	34 (32)	30 (29)	29.5 (26)	29.75 (27.5)	positive (positive)
13	21 (31.5)	23 (28)	23 (28)	23 (28)	positive (positive)
14	19.0	21.2	20.5	20.9	positive

Note: diameter of paper disk = 12.7 mm; results of previous ring test in between brackets.

All labs reported a positive result for disk H.

7. CONCLUSIONS

Table 11 gives an overview of the results obtained by the 14 laboratories.

Table 11. Overview of the results per lab and per sample (14 labs, 8 samples)

LAB	Number of correct results	Number of false positive results	Number of false negative results
1	7 (8)	0 (0)	1 (0)
2	7 (8)	0 (0)	1 (0)
3	6 (5)	0 (0)	2 (3)
4	8 (5)	0 (0)	0 (3)
5	8 (6)	0 (0)	0 (2)
6	7 (7)	0 (0)	1 (1)
7	**8 (8)**	**0 (0)**	**0 (0)**
8	8 (7)	0 (0)	0 (1)
9	8 (3)	0 (0)	0 (5)
10	6 (7)	0 (0)	2 (1)
11	7 (6)	0 (0)	1 (2)
12	7 (7)	0 (0)	1 (1)
13	**8 (8)**	**0 (0)**	**0 (0)**
14	3	0	5

Note: results of previous ring test in between brackets.

DISK	Compound	Number of correct results
A	neomycin	12
B	-	14 (14)
C	tylosin (0.5 µg)	13
D	sulfadiazine	**13** (8)
E	tylosin (0.25 µg)	8
F	sulfachloropyridazine	**13** (8)
G	ceftiofur	11 (12)
H	doxycycline	14 (12)

Note: results of previous ring test in between brackets.

Again, a large variation is observed in the results. In reality, even a larger variation can be expected since in this ring test no manipulation in the handling of kidneys is included. It is clear that still not all laboratories monitor pork carcasses at the same antimicrobial residue level.

In this ring test, no false positive results were obtained and no false negative results were obtained by labs 4, 5, 7, 8, 9 and 13.

In comparison with the previous ring test whereas 19 false negative results were obtained, 14 false negative results were obtained in this ring test with 5 false negative results generated by lab 14. So, not taking into account the bad results of lab 14 that didn't participate in the

previous ring test, it can be concluded that the ring test results are slightly improved and that remarkable better results are obtained for the group of the sulphonamides, the most occurring residues in pork meat (FASFC, results national residue plan).

Lab 14 declared not to perform the NBKT in routine with the "improved" ready-to-use Niertest Agar plates of Tritium Microbiologie (N022) that they used in this ring test. It should be emphasized that laboratories should apply the same method in a ring test as they use in routine.

No false negative results were obtained for 0.05 µg doxycycline; 1 µg neomycin, 0.5 µg tylosin, 1 µg sulfadiazine, 0.25 µg tylosin, 1.5 µg sulfachloropyridazine and 2 µg ceftiofur were considered as negative by 2, 1, 1, 6, 1 and 3 out of 14 laboratories, respectively.

It can be concluded that the "improved' ready-to-use Niertest Agar plates of Tritium Microbiologie (N022) are not yet fit for purpose, especially for the detection of sulfonamides and aminoglycosides.

Some labs still use test plates despite the fact that the control disks are indicating problems regarding the detection capability. 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 10 was not performing the test *in duplo* as requested in the legislative protocol [2] and that lab 14 did not interpret the results according to the legislative protocol [2].

Generally, the results of this ring test are slightly better than the results of the previous ring test with the exception of labs 1, 2 and 10 obtaining one extra false negative result in this ring test. Note that only labs 7 and 13 obtained excellent results in both ring tests.

Laboratories should strictly follow the prescribed protocol (volume of agar in petri dish, incubation temperature, duplicate analyses, interpretation of results, ...) . 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).

Finally, the "improved' ready-to-use Niertest Agar plates of Tritium Microbiologie (N022) are not (yet) a good alternative for Test Agar pH 7.2.

8. REFERENCES

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