



Evaluation of the TwinSensor Milk (BT00660+). Final Report (November 2010)

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T&V-ILVO Melle

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Wim Reybroeck

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Sigrid Ooghe

ABSTRACT

The TwinSensor Milk (BT00660+) is a competitive receptor test in dipstick format for the detection of residues of β -lactams (penicillins and cephalosporins) and tetracyclines in raw milk in six minutes. The TwinSensor Milk kit (type BT00660+) is a new and improved version of the TwinSensor Milk (BT00660), developed by Unisensor s.a. (Wandre, BE). With the TwinSensor Milk (BT00660) problems could occur for testing heat-treated milk, milk of species other than the cow and for milk (goat, ewe, mare) and milk with a high somatic cell count, total bacterial count or protein content.

Unisensor s.a. (Wandre, BE) worked on a new kit version resolving these problems. At the same time the test sensitivity for tetracyclines was adapted to 80-100 $\mu\text{g}/\text{kg}$.

The new version of test (type BT00660+) was validated at T&V-ILVO according to Commission Decision 2002/657/EC. The test was very specific, no interaction was noticed by compounds of antibiotic families or chemotherapeutics other than β -lactam and tetracyclines except for interference by clavulanic acid on the β -lactam channel at levels greater than 1750 $\mu\text{g}/\text{kg}$. All β -lactams with a Maximum Residue Limit (MRL) in milk were detected at their respective MRL except for nafcillin, penethamate and cefalexine (detection capability 80, 70 and 1250 $\mu\text{g}/\text{kg}$, respectively). All tetracyclines on the MRL-list could also be detected at MRL and doxycycline (not allowed for treatment of lactating cows) was detected from 50 $\mu\text{g}/\text{kg}$ on.

The repeatability of the reader and the test was very good. The test result remained reliable even when performing the TwinSensor Milk (BT00660+) with incubation times slightly different from the standard 3 + 3 min. By the use of a small device on top of the small dry-block heater the first incubation period is always correctly respected. A delay in the reading of the strips resulted in an increase of the ratio values for both channels for blank milk samples. So negative samples become more negative. The ratios for positive samples also increase but the doped samples remained positive. It's important to place the cups with receptor from the beginning in the incubator and to make the mixing with milk into the incubator so the reaction can directly start. It's also required to use a falling system 'Test stop' with open sidewall, or to remove the small system from the incubator once the dipsticks were dropped in the milk.

Also the impact of milk parameters (somatic cells, total bacterial count, fat and protein content, pH) was tested as part of the robustness testing. No interference by any milk parameter was noticed.

No significant differences were noticed in testing different milk types (raw milk, UHT-milk, sterilized milk, reconstituted milk powder or unfrozen milk). All blank milk of animal species other than the cow was all tested as negative. Just the detection of penicillin G is somewhat hampered in ewes' milk. Therefore the TwinSensor Milk (BT00660+) could also be used for testing of goat's, ewe's and mare's milk. Testing monitoring samples, no false positive or false negative results were obtained so far.

No significant differences in test capability were found between two batches of reagents of TwinSensor Milk (BT00660+).

Out of the data of this validation study it can be concluded that the present TwinSensor Milk kit (BT00660+) is a very fast, simple and reliable test for the control of residues of β -lactam and tetracycline antibiotics in tanker milk at the entrance. The test could also be used at farm level in order to prevent tanker milk contamination. The test is also giving reliable test results for heat-treated milk and milk of animal species different from the cow (goat, ewe, mare).

1. Introduction

The TwinSensor Milk is a competitive receptor test in dipstick format for the detection of residues of β -lactams (penicillins and cephalosporins) and tetracyclines in raw milk in 6 minutes. Reagents of an improved version of the kit (type BT00660+) were tested to check if the robustness of the test is improved in comparison with the first version of reagents (type BT00660).

For the first version of reagents an impact of certain milk parameters (somatic cells, total bacterial count and protein content) on the TwinSensor Milk results was noticed. Also problems occurred when testing heat-treated milk like UHT and sterilized milk and when testing milk of species other than the cow (goat, ewe, mare). Unisensor s.a. (Liège, BE) worked on a new version (type BT00660+) resolving these problems. At the same time the test sensitivity for tetracyclines was adapted to 80-100 $\mu\text{g}/\text{kg}$.

A validation study was performed at T&V-ILVO (Unit Technology & Food of the Institute for Agricultural and Fisheries Research of the Flemish Community) according to Commission Decision 2002/657/EC.

2. Test procedure

1st step: addition of 200 μl of milk to a microplate cup with receptor. Incubation during 3 min at 40°C using Heat Sensor (Unisensor s.a., Wandre, BE), a specially designed block-heater. β -lactam antibiotics possibly present in the milk will form a stable non-active complex with the specific β -lactam receptor. Tetracyclines possibly present in the milk will form a stable non-active complex with the specific tetracycline receptor. The dipstick is placed in a small device 'Test stop' on top of the incubator.

2nd step: automatic addition of a dipstick, migration and colour formation during the second incubation step of 3 min at 40°C. The bottom test line captures remaining active receptor for **β -lactams**. The upper test line captures remaining active receptor for **tetracyclines** (Figure 1).

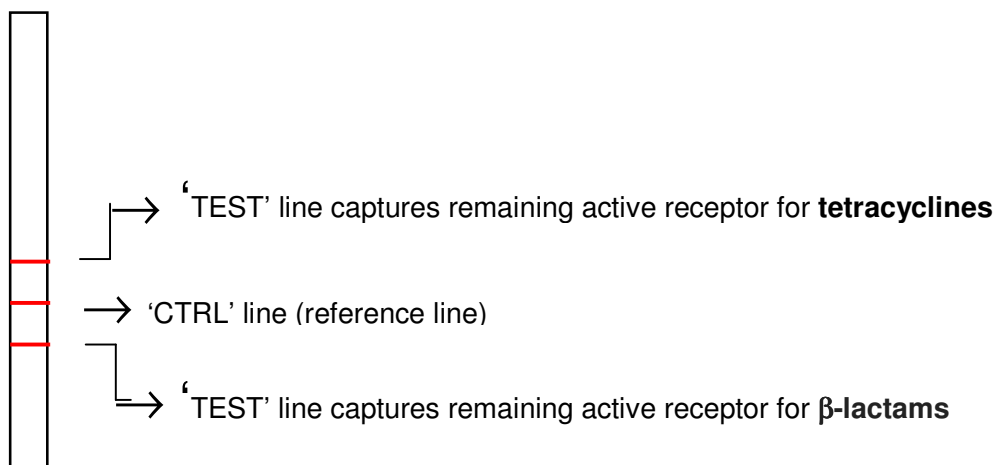


Fig. 1. Configuration of the TwinSensor dipstick.

2.1. Instrumental interpretation of the test

Reader = Read Sensor (Unisensor s.a., Liège, BE)

The reader measures the colour formation at the test line and control line positions and the RAPID software calculates the ratio's test line / control line. Calculations can be obtained based on the measurement of the area or the amplitude of colour formation. In this evaluation study all results are based on area measurements. The ratio cut-off levels are given in Table 1.

Table 1. Instrumental reading: interpretation of the test results.

Ratio (area measurement)	Interpretation
>1.10	'neg'
$0.90 < \text{ratio} \leq 1.10$	'low pos'
≤ 0.90	'pos'

2.2. Visual interpretation of the test

Visual reading of the dipsticks after incubation is also possible. The intensity of test line for β -lactam and for tetracyclines is compared to the intensity of the control line. The interpretation is shown in Figure 2.

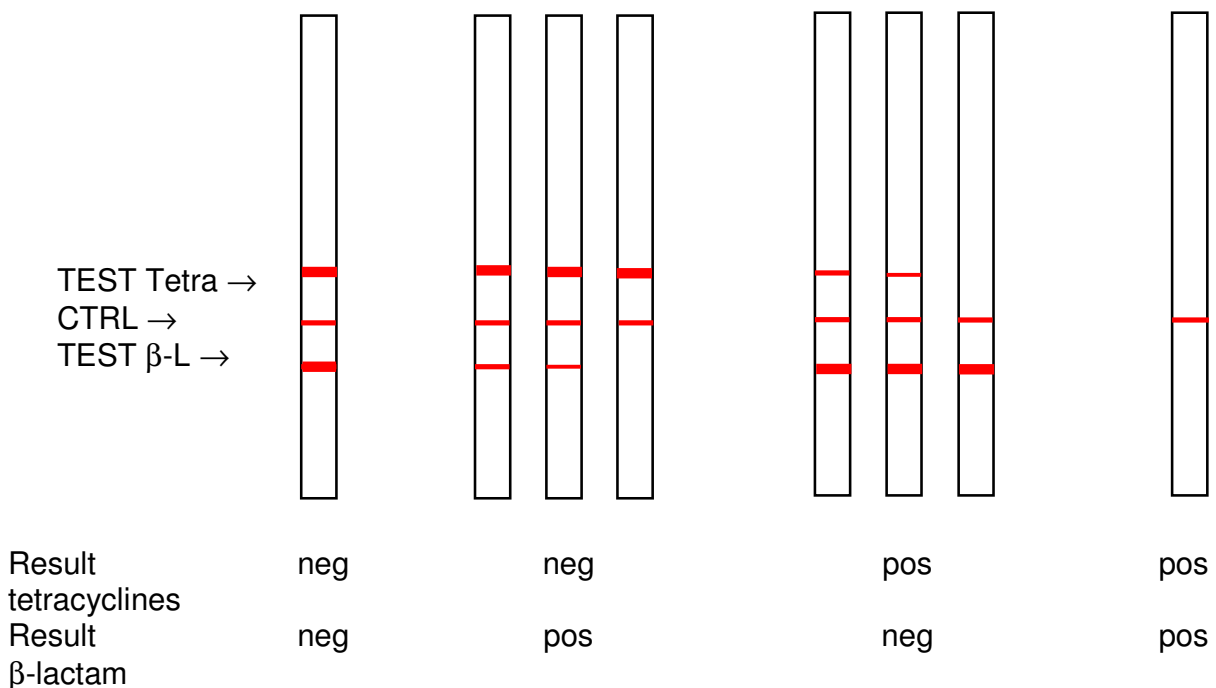


Fig. 2. Visual interpretation of the colour formation on the TwinSensor Milk (BT00660+) dipsticks.

3. Test sensitivity

Methods and material:

- Spiking of antibiotic-free (blank) raw milk with β -lactam compounds and tetracyclines. Blank milk was collected from 4 individual cows in mid-lactation not treated with veterinary drugs during the last months and giving milk with a low number of somatic cells.
- Of each compound at least 2 concentrations around the test sensitivity were tested. For the β -lactams the results from the previous study (Reybroeck and Ooghe, 2007) were used as starting point. For the tetracyclines 90 $\mu\text{g}/\text{kg}$ was used as start concentration.
- The interval between the concentrations tested for each compound was depending on the level of spiking (Table 2).

Table 2. Interval between the concentrations tested for each compound was depending on the level of spiking.

Concentration (in $\mu\text{g}/\text{kg}$)	Interval (in $\mu\text{g}/\text{kg}$)
1-10	1
10-20	2
20-50	5
50-100	10
100-250	25
250-500	50
500-1000	100
1000-5000	500

- Each concentration was tested 20 times, in a time period of at least three days.
- The test sensitivity is defined as the lowest concentration tested giving at least 19 times a positive result (upon 20 tests).
- Every day following standards were also tested:
 - 2x blank raw milk free from antimicrobials
 - 2x penicillin G 3 $\mu\text{g}/\text{kg}$ spiked in blank raw milk
 - 2x oxytetracycline 100 $\mu\text{g}/\text{kg}$ spiked in blank raw milk
- Sensitivity tests of β -lactams and tetracyclines were performed with lot 090819 (Exp. 19 August 2010) and lot 100202 (Exp.2 September 2011), respectively. The intensity of colour formation of each test line was compared to the intensity of the control line and was interpreted visually and by means of a Read Sensor reader and RAPID software. All results (reader values) were collected in a data base.

So far as available, standard material from Sigma-Aldrich was used except for ceftiofur and cefalexin (Riedel-de Haën, Bornem, Belgium), penethamate (Deltapharma s.a., Barcelona, Spain), cefquinome (Intervet International GmbH, Unterschleißheim,

Germany), cefacetrile (Novartis Animal Health Inc., Basel, Switzerland), cefalonium (Schering-Plough, Levallois-Perret, France) and for clavulanic acid (Pfizer, Kalamazoo, MI).

Results:

Details of the sensitivity testing are shown in Annex 1. A summary of the test sensitivities is given in Table 3.

Table 3. Test sensitivity (in µg/kg) of theTwinSensor Milk (BT00660+), instrumental reading (area) with cut-off ratio= 1.10. Test sensitivity defined as the lowest concentration tested giving minimum 19 positive results out of 20.

Group	Substance	MRL (µg/kg)	Test sensitivity (µg/kg)
			T&V-ILVO results
Penicillins	Penicillin G	4	3
	Ampicillin	4	4
	Amoxicillin	4	4
	Oxacillin	30	9
	Cloxacillin	30	8
	Dicloxacillin	30	7
	Nafcillin	30	80
	Penethamate	4	70
Cefalosporins	Ceftiofur	100	10
	Cefquinome	20	16
	Cefazolin	50	12
	Cephapirin	60	4
	Cefacetrile	125	16
	Cefoperazone	50	4
	Cefalexin	100	1250
	Cefalonium	20	4
Tetracyclines	Tetracycline	100	90
	Oxytetracycline	100	90
	Chlortetracycline	100	60
	Doxycycline*	---	50

MRL: Maximum Residue Limit, Regulation (EU) 37/2010 (situation on 20/01/2010);

*: not allowed to be given to lactating cows

Discussion:

TheTwinSensor Milk (BT00660+) detects all β -lactams and tetracyclines present on the EU MRL list in milk. Most of these compounds are at least 19 times out of 20 detected at MRL except for nafcillin (test sensitivity = 80 $\mu\text{g}/\text{kg}$, MRL = 30 $\mu\text{g}/\text{kg}$), penethamate (test sensitivity = 70 $\mu\text{g}/\text{kg}$, MRL = 4 $\mu\text{g}/\text{kg}$) and cefalexin (test sensitivity = 1250 $\mu\text{g}/\text{kg}$, MRL = 100 $\mu\text{g}/\text{kg}$). Doxycycline, a tetracycline without MRL in milk is detected from 50 $\mu\text{g}/\text{kg}$ on.

Note that even concentrations of β -lactams and tetracyclines in milk below the values given in Table 3 could be detected with the TwinSensor Milk (BT00660+), however not 19 times when 20 times tested.

Note also that in practice the high LOD of 70 $\mu\text{g}/\text{kg}$ for penethamate in relation to the MRL (4 $\mu\text{g}/\text{kg}$) is of no significance since penethamate is not stable in milk and is rapidly and completely hydrolysed to penicillin G and diethylaminoethanol. At 37°C and pH 7.3 (physiological conditions of cows) the half-life of penethamate in aqueous solution is 23 minutes. In tissue homogenate at 32°C half of penethamate was hydrolysed within 2 hours and at 20 hours no penethamate remained (*Anonymous*, 2000).

It's worth noting that some compounds (cephapirin, cefoperazone, ...) are already detected at concentrations several times below their respective MRL.

4. Test selectivity

Methods and material:

Testing of compounds belonging to families of antibiotics or chemotherapeutics other than β -lactams or tetracyclines (1 per family) doped at a high concentration (10x MRL or 10x MRPL in milk) in raw milk with the TwinSensor Milk (BT00660+). Testing *in duplo*. Interference of 16 β -lactam compounds on the tetracycline channel and the inference of 4 tetracyclines on the β -lactam channel was also checked.

Following compounds were used: enrofloxacin (quinolones), neomycin (aminoglycosides), lincomycin (lincosamides), clavulanic acid (β -lactamase inhibitors), colistin (polymyxins)amphenicols), chloramphenicol (florfenicol and related substances), sulfadiazine (sulfonamides), trimethoprim (diamino pyrimidine derivatives) and dapsone (other chemotherapeutics).

Results:

The ratio's for different compounds belonging to different families of antibiotics and chemotherapeutics doped at high concentration are given in Table 4.

Table 4. Ratios obtained for clavulanic acid doped in raw milk and tested with TwinSensor.

Family	Compound	Concentration doped in milk ($\mu\text{g}/\text{kg}$)	Ratio on β -Lactam channel (avg)	Ratio on Tetracycline channel (avg)
Quinolones	Enrofloxacin	1000	2.83	2.25
Aminoglycosides	Neomycin	15000	3.80	2.69
Macrolides	Erythromycin	400	3.01	2.56
Tetracyclines	Oxytetracycline	1000	3.43	0.01

Family	Compound	Concentration doped in milk (µg/kg)	Ratio on β-Lactam channel (avg)	Ratio on Tetracycline channel (avg)
Cefalosporins	Cefalonium	200	0.07	2.46
Penicillins	Penicillin G	40	0.09	2.48
Lincosamides	Lincomycin	1500	2.56	2.25
β-lactamase inhibitors	Clavulanic acid	2000	0.87	2.79
		1750	1.08	3.00
		1500	1.12	2.98
Polymyxins	Colistin	500	3.02	2.53
Chloramphenicol	Chloramphenicol	3	2.98	2.69
Sulfonamides	Sulfadiazine	1000	2.76	2.58
Diamino pyrimidine derivatives	Trimethoprim	500	2.84	2.19
Other chemotherapeutics	Dapsone	50	2.98	2.65

Discussion:

Only an interaction with clavulanic acid, a β-lactamase inhibitor, was found on the β-lactam channel of the TwinSensor Milk (BT00660+) from 1750 µg/kg and higher.

5. Test robustness

5.1. Length of incubation

Since the use of an automatic timer controlled system 'Test stop', the first incubation is always constant and respected. The impact of the length of the second incubation step was tested for the first version of reagents (type BT00660) and was not repeated for the version (type BT00660+). Performing the TwinSensor Milk (BT00660+) with a second incubation different from the standard 3 minutes has no significant impact on the ratios obtained for the β-lactam channel. For the tetracycline channel a too short second incubation could result in a drop of the ratio for blank milk (remaining negative but approaching the cut-off value). The data show that the test result remains reliable even when the second incubation is not strictly respected. Nevertheless, we still recommend to follow the test protocol as set by the kit manufacturer and as described in the kit insert.

In order to get the detection capabilities as mentioned in Table 3, it's very important to put the cups with receptor in the incubator from the beginning and to add the milk to the cups and to mix the milk with the receptor while the cups are mounted in the incubator. In this way the milk can already start to warm up and the binding reaction to start. The timer is activated once the mixing of the milk with the receptor is finished. If the mixing is performed on the laboratory table (outside the incubator), less good detection capabilities were found (data not shown) especially for compounds slower in binding to the receptor.

5.2. Repeatability of the reader

Methods and material:

The strips of blank and doped milk samples were dried to fix the colour formation. The strips were afterwards 10 times measured with a Read Sensor reader and RAPID software. The standard deviation of repeatability of the reader was calculated for both the β -lactam and tetracycline channel for 3 different levels of ratio values.

Results:

Table 6. Repeatability of the reader for the β -lactam and the tetracycline channel on different ratio levels for dried strips 10 times measured.

Milk	ratio range	n	Mean ratio		Repeatability of the test (s_r)	
			β -Lactam channel	Tetracycline channel	β -Lactam channel	Tetracycline channel
Blank milk	>1.10	10	3.52	6.43	0.10	0.15
Positive for β -lactam	<0.5	10	0.36	4.92	0.01	0.03
	0.5 -1.10	10	0.85	4.57	0.01	0.05
Positive for tetracyclines	<0.5	10	2.98	0.06	0.05	0.05
	0.5 -1.10	10	2.66	0.98	0.08	0.04

Discussion:

The repeatability of the reader is very good over the whole range of ratio values and on both channels.

5.3. Repeatability of the test

Methods and material:

Blank and doped milk samples were analysed and measured *in duplo*. The repeatability of the test was calculated for both the β -lactam and tetracycline channel for 3 different levels of ratio values (>1.1, 0-0.5 and 0.5-1.1).

Results:

Table 7. Repeatability of the test for the β -lactam and the tetracycline channel on different ratio levels.

Milk	ratio range	n	Mean ratio		Repeatability of the test (s_r)	
			β -Lactam channel	Tetracycline channel	β -Lactam channel	Tetracycline channel
Blank milk	>1.10	25	2.30	2.56	0.18	0.16
Positive for β -lactam	<0.5	10	0.34	2.84	0.05	0.14
	0.5 -1.10	10	0.65	2.91	0.04	0.12
Positive for tetracyclines	<0.5	10	2.75	0.40	0.22	0.05
	0.5 -1.10	10	2.74	0.79	0.37	0.04

Discussion:

The repeatability of the test is very good, very low standard deviation (s_r) values were obtained on both the β -lactam and the tetracycline channel. The standard deviation values are even very close to the values obtained for the reader. The repeatability for positive samples is always better than the repeatability for blank samples and for positive samples the repeatability is not significantly influenced by the ratio level.

5.4. Influence of waiting time on reader results

Methods and material:

Blank and doped milk samples were analysed and the strips were instrumentally measured directly and after 30 seconds, 1, 5 and 10 minutes. The ratio values obtained were gathered and are shown in Table 8.

Results:

Table 8. Ratios obtained when measuring strips directly after incubation and after 30 seconds, 1, 5 and 10 minutes.

Waiting time reader result									
Ratio for β-Lactam channel (T1/C)					Ratio for Tetracycline channel (T2/C)				
t=0"	t=30s	t=1min	t=5min	t=10min	t=0"	t=30s	t=1min	t=5min	t=10min
blank milk									
1.89	2.11	2.28	2.34	2.27	1.54	1.84	2.08	2.38	2.40
1.98	2.04	2.22	2.78	2.69	1.48	1.71	1.94	2.60	2.88
2.37	2.32	2.57	2.71	2.82	1.93	1.96	2.23	2.54	2.74
2.00	2.28	2.35	2.81	2.88	1.54	2.01	2.11	2.76	2.99
milk doped with 3 μg/kg penicillin G									
0.58	0.60	0.66	0.78	0.74	1.78	2.07	2.36	3.20	3.34
0.54	0.63	0.56	0.69	0.67	1.60	1.95	1.91	3.03	3.27
0.50	0.52	0.55	0.63	0.63	1.78	2.06	2.12	2.96	3.29
0.49	0.51	0.61	0.66	0.53	1.46	1.80	1.95	2.29	2.36
milk doped with 100 μg/kg oxytetracycline									
2.22	2.33	2.41	2.99	3.06	0.42	0.51	0.56	0.87	0.97
2.19	2.39	2.33	2.87	3.15	0.40	0.50	0.53	0.89	1.06
2.09	2.26	2.19	2.83	2.97	0.40	0.50	0.55	0.97	1.06
1.83	1.93	2.05	2.47	2.59	0.36	0.42	0.44	0.76	0.86

Discussion:

A delay in the reading of the strips results in an increase of the ratio values, especially for blank milk samples. So negative samples become more negative; the increase of the ratio for positive samples remains limited so the loss in sensitivity is not dramatic. On the tetracycline channel the impact is most pronounced: the ratios of blank and tetracycline-positive samples nearly double after 10 minutes. So when delaying the reading, the TwinSensor Milk (BT00660+) test becomes less sensitive for tetracyclines. As long as the strips are read within one minute after the end of the incubation, no real impact can

be expected. Longer delay in reading of the strips is of no importance for negative milk samples but results in a small loss in sensitivity for the tetracycline detection. However, a concentration of oxytetracycline at MRL (100 µg/kg) was still screened as positive with a reading delayed for 10 minutes.

Despite the results we still recommend to read the strips directly after the test is finished.

5.5. Influence of falling device ‘Test stop’

A prototype of the ‘Test stop’ was used. This system is placed on top of the incubator and regulates the falling of the strips into the cups at the end of the first incubation period. Two protocols were tested: the ‘Test stop’ remaining on top of the incubator after addition of the strips to the cups and removal of the ‘Test stop’ device once his work done. Results in detection capabilities for tetracyclines are shown in Table 9.

Table 9. Detection capabilities for tetracyclines with the ‘Test stop’ remaining on the incubator and the small device removed once the strips were added to the milk and receptor in the cups.

Group	Substance	Test sensitivity (µg/kg)	
		Test stop remaining	Test stop removed
Tetracyclines	Tetracycline	125	90
	Oxytetracycline	100	90
	Chlortetracycline	80	60
	Doxycycline	70	50

An influence of the ‘Test stop’ device was remarked on the detection capabilities. Especially the detection of tetracyclines was better after the removal of the ‘Test stop’. This could perhaps be explained by the fact that the funnels of the ‘Test stop’ are keeping the heat of the incubator (chimney effect) what is influencing the flow and the reaction of the reagents.

So, it is required to use a falling system ‘Test stop’ with open sidewall, or to remove the small system from the incubator once the dipsticks were dropped in the milk.

5.6. Milk influences

5.6.1. Somatic cell count

Methods and material:

Normal milk samples and milk samples with a high somatic cell count (> 1.10⁶ per ml) were analysed and the ratios obtained were compared in order to study the impact of the somatic cell count on the TwinSensor Milk (BT00660+) result. The milk samples with a high number of somatic cells were selected at the milk control station based on Fossomatic 5000 (FOSS, Hillerød, DK) measurements.

Results:

Table 10. Ratios obtained for normal milk samples and for milk samples with a high somatic cell count ($> 1.10^6$ per ml).

	n	Ratio for β -Lactam channel			Ratio for Tetracycline channel		
		min	max	mean	min	max	mean
Normal milk							
Blank milk	4	2.29	2.79	2.56	2.25	2.55	2.42
Milk + 3 $\mu\text{g/kg}$ penicillin G	4	0.32	0.58	0.47	2.15	2.86	2.46
Milk + 100 $\mu\text{g/kg}$ oxytetracycl.	4	2.47	2.57	2.53	0.45	0.75	0.61
Milk with a high somatic cell count ($> 1.10^6$ per ml)							
Blank milk	30	1.60	2.89	2.33	1.90	3.37	2.68
Milk + 3 $\mu\text{g/kg}$ penicillin G	10	0.43	0.92	0.73	2.33	2.99	2.62
Milk + 100 $\mu\text{g/kg}$ oxytetracycl.	10	2.01	2.89	2.43	0.13	0.50	0.29

Discussion:

The somatic cell count of the milk is not influencing the result of the TwinSensor Milk (BT00660+). These results proof the improvements made by the kit manufacturer to this version of reagents.

5.6.2. Total bacterial count

Methods and material:

Normal milk samples and milk samples with a high total bacterial count ($>5.10^5$ CFU per ml) were analysed and the ratios obtained were compared in order to study the impact of the total bacterial count on the TwinSensor Milk (BT00660+) result. The milk samples with a high total bacterial count were obtained by keeping normal milk samples during 4-6 hours at room temperature. The final bacterial count were determined by performing a spiral plate count (Eddy Jet, IUL sa, Barcelona, Spain) on Plate count agar plates after 3 days incubation at 30°C.

Results:

Table 11. Ratios obtained for normal milk samples and for milk samples with a high total bacterial count ($>5.10^5$ CFU per ml).

	n	Ratio for β -Lactam channel			Ratio for Tetracycline channel		
		min	max	mean	min	max	mean
Normal milk (samples before incubation)							
Blank milk	20	2.24	3.16	2.75	2.15	3.36	2.79
Milk + 3 $\mu\text{g/kg}$ penicillin G	4	0.48	0.94	0.66	2.68	3.19	2.97
Milk +100 $\mu\text{g/kg}$ oxytetracycline	4	3.03	3.16	3.09	0.78	1.03	0.90
Milk with a high total bacterial count ($>5.10^5$ CFU per ml) (samples after incubation)							
Blank milk	20	2.48	3.35	2.92	2.28	3.27	2.81
Milk + 3 $\mu\text{g/kg}$ penicillin G	10	0.37	1.88	0.88	0.01	2.99	2.40
Milk +100 $\mu\text{g/kg}$ oxytetracycline	10	1.62	3.94	3.13	0.13	0.54	0.35

Discussion:

The bacterial count of the milk is not influencing the result of the TwinSensor Milk (BT00660+). Tetracyclines are even slightly better detected in milk with a high total bacterial count.

5.6.3. Fat content

Methods and material:

Normal milk samples and milk samples with a low (< 2 g per 100 ml) or a high (>6 g per 100 ml) fat content were analysed and the ratios obtained were compared in order to study the impact of the fat content on the TwinSensor Milk (BT00660+) result. The milk samples with a low fat content were partly obtained by removal of the fat layer after 8 minutes centrifuging of the samples. Part of the samples were milk samples with a natural low fat content. The milk samples with a high fat content were milk samples with a natural extreme fat content and were selected at the milk control station based on infrared spectroscopic results with a MilcoScan 4000 (FOSS, Hillerød, DK).

Results:

Table 11. Ratios obtained for normal milk samples and for milk samples with a low (< 2 g per 100 ml) or a high (> 5 g per 100 ml) fat content.

	n	Ratio for			Ratio for		
		β-Lactam channel			Tetracycline channel		
		min	max	mean	min	max	mean
Normal milk							
Blank milk	2	2.34	2.36	2.35	2.34	2.43	2.39
Milk + 3 µg/kg penicillin G	4	0.50	0.70	0.64	2.53	3.32	2.90
Milk + 100 µg/kg oxytetracycline*	6	2.45	2.74	2.59	1.18	1.68	1.38
Milk with a low fat content (< 2 g per 100 ml)							
Blank milk	10	2.47	3.23	2.70	2.82	3.44	3.10
Milk + 3 µg/kg penicillin G	10	0.60	0.95	0.76	2.24	3.38	2.93
Milk + 100 µg/kg oxytetracycline*	10	2.50	3.19	2.82	1.08	1.83	1.49
Normal milk							
Blank milk	2	2.29	2.79	2.56	2.25	2.55	2.42
Milk + 3 µg/kg penicillin G	4	0.32	0.58	0.47	2.15	2.86	2.46
Milk + 100 µg/kg oxytetracycline	4	2.47	2.57	2.53	0.45	0.75	0.61
Milk with a high fat content (> 6 g per 100 ml)							
Blank milk	10	1.23	3.60	2.48	1.23	3.41	2.43
Milk + 3 µg/kg penicillin G	10	0.38	1.46	0.59	1.81	2.53	2.26
Milk + 100 µg/kg oxytetracycline	10	1.55	3.39	2.52	0.11	0.69	0.37

*: tested with 'Test stop' remaining on the incubator during the second incubation

Discussion:

The fat content of the milk is not influencing the result of the TwinSensor Milk (BT00660+). A high fat content can hamper the flow on the dipstick. So the colour development of the line on the tetracycline channel can be delayed resulting in slightly lower ratio values (more positive).

5.6.4. Protein content

Methods and material:

Normal milk samples and milk samples with a low (< 2.5 g per 100 ml) or a high (> 4 g per 100 g) protein content were analysed and the ratios obtained were compared in order to study the impact of the protein content on the TwinSensor Milk (BT00660+) result. The milk samples with a low and a high protein content are milk samples with a natural extreme protein content and were selected at the milk control station based on infrared spectroscopic results with a MilcoScan 4000.

Results:

Table 12. Ratios obtained for normal milk samples and for milk samples with a low (< 2.5 g per 100 ml) or a high (> 4 g per 100 ml) protein content.

	n	Ratio for β-Lactam channel			Ratio for Tetracycline channel		
		min	max	mean	min	max	mean
Normal milk							
Blank milk	6	2.33	3.24	2.84	2.37	3.23	2.83
Milk + 3 µg/kg penicillin G	6	0.32	0.60	0.41	1.91	3.33	2.68
Milk + 100 µg/kg oxytetracycline*	6	2.39	2.96	2.64	1.02	1.19	1.12
Milk with a low protein content (< 2.5 g/100 ml)							
Blank milk	10	1.89	3.45	2.93	2.41	3.19	2.87
Milk + 3 µg/kg penicillin G	10	0.38	0.92	0.61	2.60	3.41	3.13
Milk + 100 µg/kg oxytetracycline*	10	3.02	3.63	3.30	0.27	1.25	0.98
Normal milk							
Blank milk	2	2.29	2.79	2.56	2.25	2.55	2.42
Milk + 3 µg/kg penicillin G	4	0.32	0.58	0.47	2.15	2.86	2.46
Milk + 100 µg/kg oxytetracycline*	4	2.47	2.57	2.53	0.45	0.75	0.61
Milk with a high protein content (> 4 g/100 ml)							
Blank milk	10	1.87	3.09	2.32	2.05	2.75	2.41
Milk + 3 µg/kg penicillin G	10	0.41	0.97	0.65	2.23	2.80	2.57
Milk + 100 µg/kg oxytetracycline*	10	1.97	2.82	2.33	0.38	1.16	0.63

*: tested with 'Test stop' remaining on the incubator during the second incubation

Discussion:

The protein content of the milk is not significantly influencing the result of the TwinSensor Milk (BT00660+). Residues of oxytetracycline are even slightly better detected in milk with a low protein content.

5.6.5. pH

Methods and material:

Milk samples with a normal pH (6.7 - 6.9) and milk samples with a low pH (6.0) or a high pH (7.5) were analysed and the ratios obtained were compared in order to study the impact of the milk pH on the TwinSensor Milk (BT00660+) result. To milk samples with a

normal pH respectively 1 M HCl or 1 M NaOH was added. Afterwards the pH of the milk was brought exactly to 6.0 and 7.5 by fine-tuning with 0.1 M HCl and/or 0.1 M NaOH.

Results:

Table 13. Ratios obtained for milk samples with a normal pH and for milk samples with a low pH (6.0) or a high pH (7.5).

	n	Ratio for			Ratio for		
		β-Lactam channel			Tetracycline channel		
		min	max	mean	min	max	mean
Milk with a normal pH							
Blank milk	10	2.74	3.91	3.40	2.90	3.52	3.36
Milk + 3 µg/kg penicillin G	4	0.46	0.50	0.49	3.48	3.66	3.55
Milk + 100 µg/kg oxytetracycline*	4	3.26	3.60	3.44	1.24	1.53	1.41
Milk with a low pH (6.0)							
Blank milk	10	1.82	3.09	2.30	2.18	3.56	2.69
Milk + 3 µg/kg penicillin G	10	0.30	0.88	0.66	2.91	3.40	3.20
Milk + 100 µg/kg oxytetracycline*	10	2.62	3.33	3.02	0.25	0.64	0.46
Milk with a normal pH							
Blank milk	10	2.74	3.91	3.40	2.90	3.52	3.36
Milk + 3 µg/kg penicillin G	4	0.46	0.50	0.49	3.48	3.66	3.55
Milk + 30 µg/kg oxytetracycline*	4	3.26	3.60	3.44	1.24	1.53	1.41
Milk with a high pH (7.5)							
Blank milk	10	2.43	3.71	3.10	1.92	3.37	2.57
Milk + 3 µg/kg penicillin G	10	0.43	0.72	0.55	2.33	3.61	2.90
Milk + 100 µg/kg oxytetracycline*	10	2.14	4.04	3.02	1.31	2.00	1.60

*: tested with 'Test stop' remaining on the incubator during the second incubation

Discussion:

A low (pH 6.0) or a high (pH 7.5) pH of milk is not influencing the result of the TwinSensor Milk (BT00660+). Oxytetracycline is better detected in milk with a low pH.

5.6.6. Type of milk

Methods and material:

Raw milk, UHT milk, sterilized milk, reconstituted milk powder and unfrozen milk were analysed in order to check if the TwinSensor Milk (BT00660+) is a suitable test for these types of milk.

Results:

Table 14. Ratios obtained for raw milk, UHT milk, sterilized milk, reconstituted milk powder and unfrozen milk.

	n	Ratio for			Ratio for		
		β-Lactam channel			Tetracycline channel		
		min	max	mean	min	max	mean
Raw milk							
Blank milk	6	2.06	2.68	2.36	2.28	2.67	2.47
Milk + 3 µg/kg penicillin G	6	0.44	0.66	0.54	2.07	2.66	2.43
Milk + 100 µg/kg oxytetracycline	6	2.71	3.30	2.93	0.10	0.80	0.47
UHT milk							
Blank milk	20	2.34	3.74	2.90	1.68	3.27	2.34
Milk + 3 µg/kg penicillin G	10	0.60	0.85	0.74	1.90	2.70	2.32
Milk + 100 µg/kg oxytetracycline	10	1.92	3.43	2.64	0.44	0.86	0.68
Raw milk							
Blank milk	6	2.50	3.17	2.79	2.37	2.96	2.60
Milk + 3 µg/kg penicillin G	6	0.41	0.86	0.64	2.35	3.41	2.78
Milk + 100 µg/kg oxytetracycline	6	2.71	3.30	2.93	0.10	0.80	0.47
Sterilized milk							
Blank milk	20	2.29	3.16	2.71	1.52	2.54	2.09
Milk + 3 µg/kg penicillin G	10	0.41	1.11	0.78	1.60	3.06	2.29
Milk + 100 µg/kg oxytetracycline	10	2.45	3.13	2.75	0.14	0.61	0.40
Raw milk							
Blank milk	6	2.50	3.17	2.79	2.37	2.96	2.60
Milk + 3 µg/kg penicillin G	6	0.41	0.86	0.64	2.35	3.41	2.78
Milk + 100 µg/kg oxytetracycline	6	2.71	3.30	2.93	0.10	0.80	0.47
Reconstituted milk powder							
Blank milk	20	2.64	3.52	3.00	1.98	3.03	2.42
Milk + 3 µg/kg penicillin G	10	0.48	0.84	0.64	1.78	2.57	2.27
Milk + 100 µg/kg oxytetracycline	10	2.60	3.29	3.02	0.45	0.89	0.61
Raw milk							
Blank milk	6	2.50	3.17	2.79	2.37	2.96	2.60
Milk + 3 µg/kg penicillin G	6	0.41	0.86	0.64	2.35	3.41	2.78
Milk + 100 µg/kg oxytetracycline	6	2.71	3.30	2.93	0.10	0.80	0.47
Unfrozen milk							
Blank milk	20	2.38	3.09	2.78	2.37	3.20	2.84
Milk + 3 µg/kg penicillin G	10	0.42	0.64	0.55	2.35	3.05	2.66
Milk + 100 µg/kg oxytetracycline	10	2.38	2.89	2.63	0.54	0.79	0.67

Discussion:

TwinSensor Milk (BT00660+) is a test developed for the testing of raw cow's milk on the presence of residues of β-lactam and tetracycline antibiotics. However the test can also be used for the testing of heat-treated milk and milk powder. No influence was noticed due to storage of the milk samples at <-18°C.

5.6.7. Milk from animal species other than the cow

Methods and material:

Raw goats' milk, raw ewes' milk and raw mares' milk samples were analysed to test if the TwinSensor Milk (BT00660+) is a suitable test for these types of milk coming from animal species other than the cow.

Results:

Table 15. Ratios obtained for cow's milk (reference), goats' milk, ewes' milk and mares' milk.

	n	Ratio for β -Lactam channel			Ratio for Tetracycline channel		
		min	max	mean	min	max	mean
Cows' milk							
Blank milk	6	2.50	3.17	2.79	2.37	2.96	2.60
Milk + 3 μ g/kg penicillin G	6	0.41	0.86	0.64	2.35	3.41	2.78
Milk + 100 μ g/kg oxytetracycline	6	2.71	3.30	2.93	0.10	0.80	0.47
Ewes' milk							
Blank milk	20	2.02	3.58	2.63	1.30	3.28	2.17
Milk + 3 μ g/kg penicillin G	10	0.58	1.98	1.00	1.77	3.04	2.15
Milk + 100 μ g/kg oxytetracycline	10	1.84	3.31	2.61	0.33	0.92	0.65
Cows' milk							
Blank milk	6	2.44	3.24	2.90	2.44	3.24	2.95
Milk + 3 μ g/kg penicillin G	6	0.44	0.71	0.56	2.35	3.26	2.78
Milk + 100 μ g/kg oxytetracycline	6	2.56	2.95	2.78	0.79	0.91	0.85
Goats' milk							
Blank milk	20	2.25	3.60	2.97	1.55	2.97	2.38
Milk + 3 μ g/kg penicillin G	10	0.71	1.20	0.98	2.11	2.97	2.52
Milk + 100 μ g/kg oxytetracycline	10	2.40	3.49	2.97	0.16	0.39	0.32
Cows' milk							
Blank milk	2	2.63	2.82	2.73	2.95	3.30	3.13
Milk + 3 μ g/kg penicillin G	6	0.40	0.46	0.43	3.01	3.33	3.11
Milk + 100 μ g/kg oxytetracycline	6	2.20	2.65	2.49	0.75	0.90	0.84
Mares' milk							
Blank milk	20	3.34	4.96	3.92	2.24	4.29	3.17
Milk + 3 μ g/kg penicillin G	10	0.38	1.41	1.08	2.66	3.80	3.42
Milk + 100 μ g/kg oxytetracycline	10	3.48	3.91	3.69	0.03	0.21	0.12

Discussion:

TwinSensor Milk (BT00660+) is a test developed for the testing of cows' milk on the presence of residues of β -lactam and tetracycline antibiotics. When applying the test on milk of other animal species, no problems were encountered. Just the detection of penicillin G is somewhat hampered in ewes' milk. The TwinSensor Milk (BT00660+) is also a suitable test to screen milk of species other than the cow (goat, ewe, mare).

5.7. Test for false positive/false negative results

Methods and material:

425 Farm and 181 truck milk samples were analysed with TwinSensor Milk (BT00660+) and other microbiological and receptor screening tests.

The test was also integrated in the monitoring of dairy samples in the framework of the national residue plan. 372 Farm milk, 181 truck milk, 32 consumption milk samples and 16 milk powders were analysed with TwinSensor Milk (BT00660+). The same samples were also tested by Delvotest SP-NT (DSM Food Specialties, Delft, NL), *Bacillus cereus*-test, *Escherichia coli*-test and β eta-s.t.a.r. (Neogen Corporation, Lansing, MI).

Results and discussion:

All 425 farm samples tested negative on both β -lactam and tetracycline channel of the TwinSensor Milk (BT00660+). So no false positive results were obtained.

5.8. Reagent influence (batch differences)

Methods and material:

Following samples were analysed at the same time with 2 different batches of TwinSensor Milk (BT00660+):

- Blank milk (antibiotic-free raw milk) (20 samples)
- Raw milk doped with 3 μ g/kg penicillin G (10 samples)
- Raw milk doped with 100 μ g/kg oxytetracycline (10 samples)
- Raw milk doped with 4 μ g/kg cefalonium (10 samples)

As TwinSensor Milk (BT00660+) reagents lot 090821 (Exp. August 21, 2010) and lot 100113 (Exp. January 13, 2011) were used.

Results:

Table 16. Ratios obtained when testing negative and positive milk samples with reagents from different batches.

	Batch 090821						Batch 100113					
	β -Lactam			Tetracyclines			β -Lactam			Tetracyclines		
(n=20)	min	max	mean	min	max	mean	min	max	mean	min	max	mean
blank	2.21	3.22	2.83	1.72	2.80	2.46	2.18	3.15	2.66	2.25	3.79	3.10
pen 3 μ g/kg	0.53	0.77	0.65	1.59	2.79	2.18	0.62	0.86	0.75	2.18	3.62	2.93
otc 100 μ g/kg	2.28	3.16	2.75	0.25	0.55	0.41	2.16	2.96	2.61	0.25	0.42	0.33
cefalo 4 μ g/kg	0.47	0.83	0.59	1.65	2.73	2.14	0.65	0.93	0.76	2.27	2.99	2.90

Pen: penicillin G, otc: oxytetracycline, cefalo: cefalonium

Discussion:

No significant differences in detection capability were noticed between both lots of reagents.

6. Interlaboratory testing

6.1. International proficiency testing

T&V-ILVO participated end of May 2009 in an international proficiency study for the analysis of antibiotic residues in raw milk organised by the EU Community reference laboratory of AFSSA (Fougères, FR).

Results and discussion:

The proficiency test consisted out of 14 blind coded milk samples: 2 blank milk samples, 2 samples spiked with respectively 4 µg/kg penicillin G, 30µg/kg cloxacillin, 50 µg/kg cefquinome, 20 µg/kg cephalonium, 100 µg/kg and 200 µg/kg oxytetracycline. The TwinSensor Milk (BT00660+) screened the blank milk samples as negative and all spiked samples as positive.

6.2. National ring trial

Methods and material:

T&V-ILVO is organizing twice a year a national ring trial for the Belgian dairy industry regarding the detection of residues of antibiotics in milk by microbiological and rapid tests. In the ring trials of 29 October 2009 and 22 April 2010 the TwinSensor Milk (BT00660+) was also integrated as rapid test.

Results and discussion:

The national ring trial of 29 October 2009 consisted out of 8 blind coded milk samples: milk spiked with 3 µg/kg penicillin G, 4 µg/kg penicillin G (2 samples), 4 µg/kg ampicillin, 30 µg/kg cloxacillin, 20 µg/kg cefquinome, 100 µg/kg oxytetracycline and 100 µg/kg chlortetracycline, respectively. The TwinSensor Milk (BT00660+) screened all β-lactams and 100 µg/kg chlortetracycline as positive. The milk spiked with 100 µg/kg oxytetracycline was screened as negative (visual reading positive).

The national ring trial of 22 April 2010 consisted out of 8 blind coded milk samples: milk spiked with 3 µg/kg penicillin G, 4 µg/kg penicillin G, 30 µg/kg cloxacillin, 30 µg/kg nafcillin, 15 µg/kg ceftiofur, 20 µg/kg doxycycline, 100 µg/kg tetracycline, respectively and 1 blank milk sample. The TwinSensor Milk (BT00660+) screened all β-lactams as positive, except for nafcillin as could be expected. The milk spiked with 100 µg/kg tetracycline was screened as positive while the 20 µg/kg doxycycline tested negative. So all results are in line with the detection capabilities mentioned in Table 3.

7. Daily control samples

Methods and material:

Zero-setting of the Read Sensor reader is daily performed by means of a Blank dipstick (Batch 090610, Exp. June 10, 2010). The reader was daily checked by measuring a Check dipstick (Batch 090209, Exp. June 10, 2010). The ratio values are presented in Figure 3.

Following control samples were daily twice analysed with the TwinSensor Milk (BT00660+):

- Blank milk (antibiotic-free raw milk)
- Raw milk doped with 3 µg/kg penicillin G
- Raw milk doped with 100 µg/kg oxytetracycline

The results obtained for the control samples are presented in Figure 4.

There are also negative and positive freeze-dried milk standards from Unisensor s.a. available for a daily check of the reagents and reader instrument. The data obtained for Negative control and Positive control standards are shown in Figure 5.

Results and discussion:

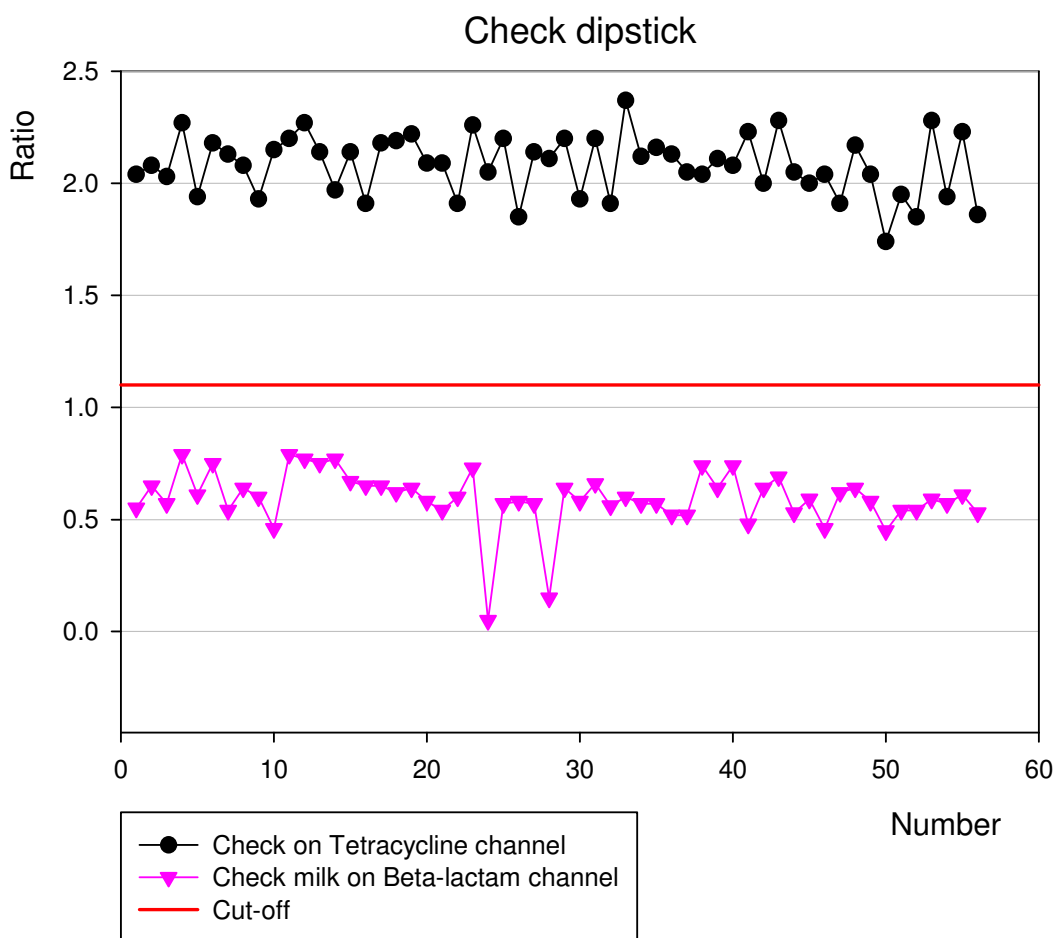


Fig. 3. Results (ratio) for the check dipstick.

In general very stable ratio values were obtained for the check dipsticks over the test period on both test channels. The ratio values for the Check dipstick on the β-lactam test line ranged from 0.05 to 0.79 (mean = 0.59) with a standard deviation of 0.13; the ratio values for the Check dipstick on the tetracycline test line ranged from 1.74 to 2.37 (mean = 2.08) with a standard deviation of 0.13.

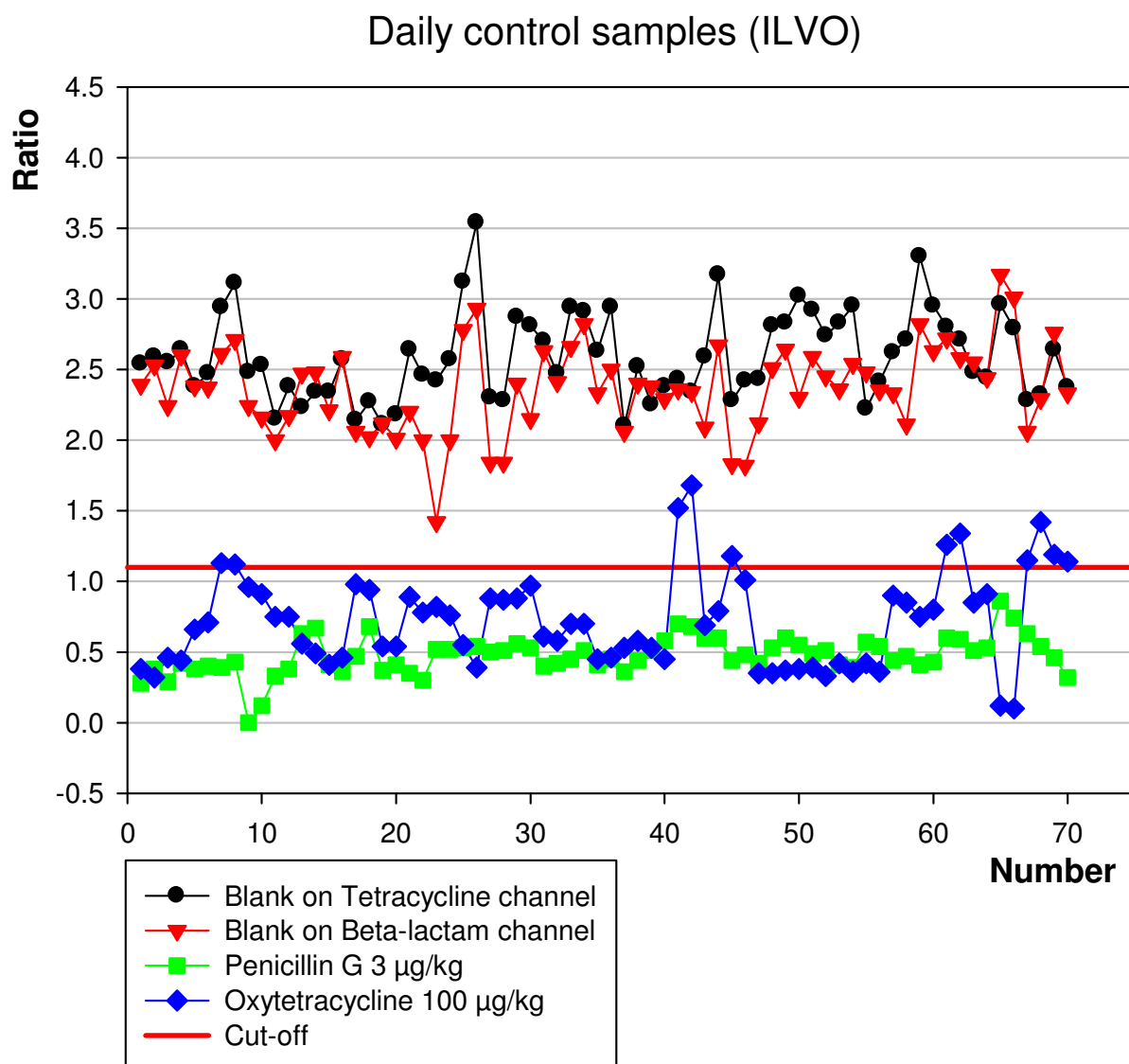


Fig. 4. Results (ratio) for the control samples (ILVO), tested with 'Test stop' remaining on the incubator during the second incubation.

For the daily fresh prepared control samples following standard deviations were obtained: blank milk on the β -lactam channel: 0.31 (mean = 2.37), blank milk on the Tetracycline channel: 0.31 (mean = 2.59), penicillin 3 $\mu\text{g}/\text{kg}$ on the β -lactam channel: 0.13 (mean = 0.48), oxytetracycline 100 $\mu\text{g}/\text{kg}$ on the Tetracycline channel: 0.33 (mean = 0.72). Eleven times the checks of the tetracycline 100 $\mu\text{g}/\text{kg}$ control sample were giving a negative result, mostly with ratio values <1.5 . The impact of the Test stop remaining on top of the incubator on the detection capability, especially on the tetracycline channel, was detected half-way the validation study. For the daily control samples it was decided to keep to the start protocol to avoid an impact on this figure. Tetracyclines are known to bind on the Ca present in the milk. The negative results for the oxytetracycline 100 $\mu\text{g}/\text{kg}$ could perhaps been generated with standards not directly tested after preparation, resulting in partly binding to Ca and some degradation. The

extremely low ratio's obtained for sample 65 and 56 could not directly be explained; an error in spiking could not be excluded.

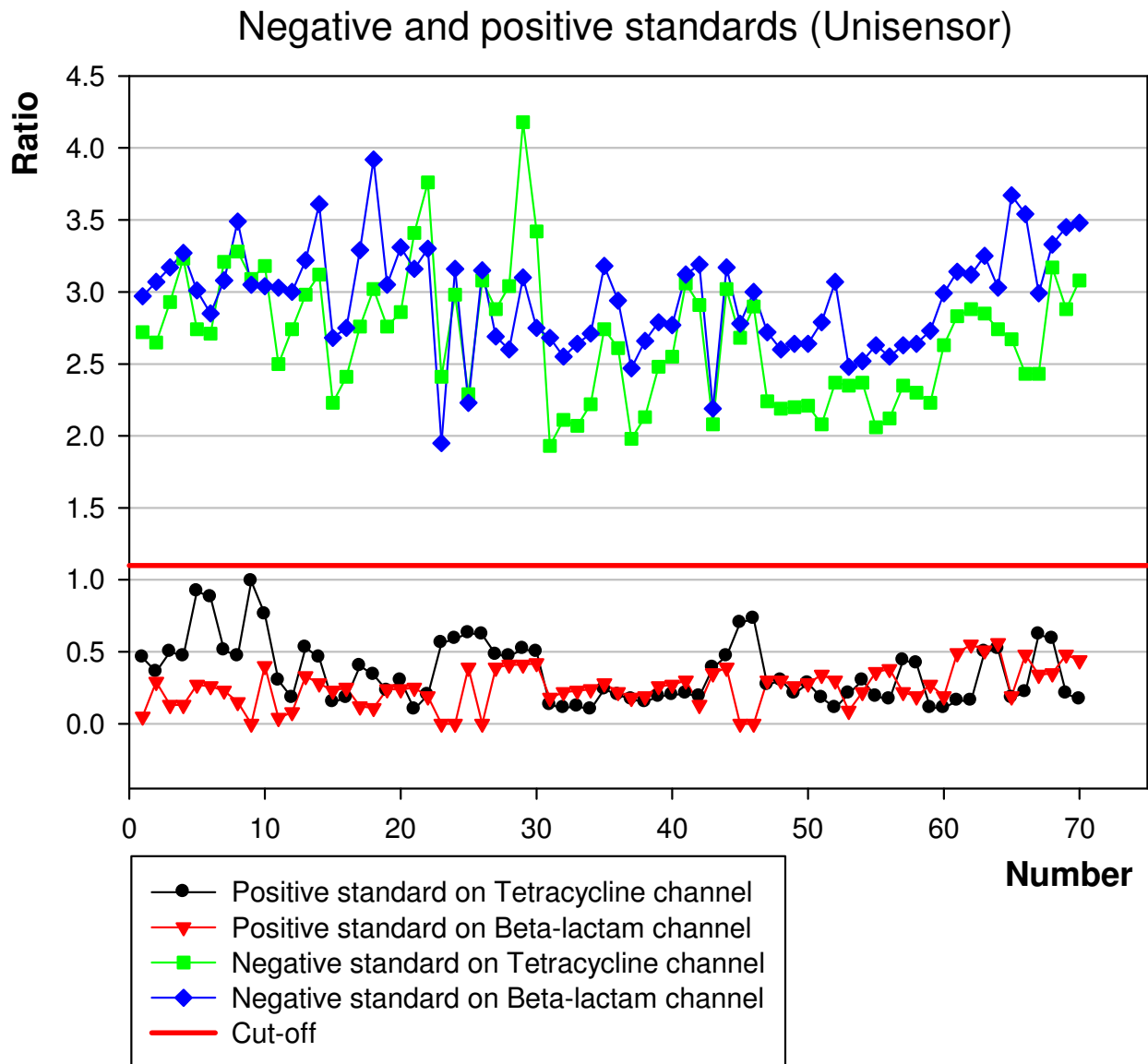


Fig. 5. Results (ratio) obtained for the negative and positive standards, (.), tested with 'Test stop' remaining on the incubator during the second incubation.

The results for the reconstituted negative and positive standards were satisfactory. The positive standard always tested positive on both β -Lactam and Tetracycline channel. Following standard deviations were obtained: negative control on the β -lactam channel: 0.36 (mean = 2.95), negative control on the Tetracycline channel: 0.45 (mean = 2.68), positive control on the β -lactam channel: 0.14 (mean = 0.26), positive control on the Tetracycline channel: 0.22 (mean = 0.36). It's worth noting that the variations of the ratio values obtained for the negative standards are similar to values for the negative control milk samples despite the fact that the blank raw milk (control samples) were different in composition every two days.

FINAL CONCLUSION

The TwinSensor Milk (BT00660+) is a fast, simple and reliable test for the control of residues of β -lactam and tetracycline antibiotics in tanker milk at the entrance.

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REFERENCES

Anonymous (2000) Committee for Veterinary Medicinal Products: Penethamate (summary report) The European Agency for the Evaluation of Medicinal Plants, Veterinary Medicines Evaluation Unit, London, United Kingdom, 1-3.

Anonymous (2009) Proficiency testing for the analysis of antibiotic residues in raw milk. Final report version 1 published the 22nd December 2009. Study Code: 09-ZU. Community Reference Laboratory for antimicrobial residues in food of animal origin, AFSSA Fougères, France. 51 pp.

Council Regulation N°2377/90 of 26 June 1990, Off. J. Eur. Communities, L224, 1-8.

Council Commission Decision N° 2002/657/EC, Off.J. Communities L221, 1-8.

Council Regulation (EU) N° 37/2010 of 22 December 2009, Off. J. Eur. Union, L15, 1-72.

Kit insert Unisensor s.a.

Ooghe, S., Reybroeck, W. Report ring trial TwinSensor. 2006, 1-18.

Ooghe, S., Reybroeck, W. Rapport ringonderzoek antibiotica: microbiologische testen en sneltesten. 29 oktober 2009, 1-25.

Reybroeck, W., Ooghe, S. Validation of the TwinSensor Milk for the rapid screening of β -lactams and tetracyclines in milk. Oral presentation at the 5th International Symposium on Hormone and Veterinary Drug Residue Analysis, Antwerp, Belgium, May 16-19, 2006. Abstract in Abstract book p. 26.

Reybroeck, W., Ooghe, S. Evaluation of the TwinSensor Milk. Final report, November 9th 2007, 1-26.

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ANNEX 1. DETAILS OF THE SENSITIVITY TESTING.

Substance	Conc. (µg/kg)	n	Reader Values β-lactam			Reader Values Tetracyclines			Visual pos	reader pos
			Lowest T1/C	Highest T1/C	Mean T1/C	Lowest T2/C	Highest T2/C	Mean T2/C		
Penicillin G	2	20	0.72	1.54	1.05	2.43	4.24	3.09	12	12
	3	20	0.06	0.57	0.41	2.34	5.30	3.62	20	20
Ampicillin	3	20	1.10	1.66	1.42	2.64	3.60	3.17	1	1
	4	40	0.64	1.14	0.90	0.79	3.76	2.90	38	38
	5	20	0.53	1.02	0.77	2.28	3.52	3.05	20	20
Amoxicillin	2	2	1.83	1.89	1.86	3.85	3.94	3.90	0	0
	3	20	0.83	1.49	1.18	2.32	3.63	3.03	6	6
	4	20	0.61	1.20	0.87	2.32	3.51	2.99	19	19
	5	20	0.46	1.07	0.71	2.33	3.43	2.94	20	20
Cloxacillin	5	2	1.14	1.37	1.26	3.26	3.30	3.28	0	0
	6	6	0.90	1.21	1.08	2.65	3.32	3.12	18	18
	7	20	0.79	1.34	1.01	2.39	4.41	3.24	15	15
	8	20	0.61	1.01	0.80	2.63	4.13	3.34	20	20
Nafcillin	50	4	1.35	1.53	1.48	3.01	3.41	3.23	0	0
	60	4	1.09	1.29	1.19	2.50	2.93	2.75	1	1
	70	8	0.99	1.19	1.06	2.19	3.10	2.52	6	6
	80	20	0.74	1.08	0.87	2.20	3.35	2.70	20	20
Dicloxacillin	5	2	1.03	1.41	1.22	2.92	3.67	3.30	1	1
	6	20	0.77	1.16	0.96	2.19	3.91	3.07	18	18
	7	20	0.37	1.05	0.71	2.31	3.83	2.98	20	20
	8	4	0.73	0.83	0.78	3.03	3.34	3.19	4	4
Oxacillin	8	20	0.93	1.19	1.04	2.34	3.31	2.84	18	18
	9	20	0.81	1.12	0.98	2.47	3.46	3.00	19	19
	10	20	0.66	1.09	0.86	2.28	3.57	3.09	20	20
	16	2	0.55	0.56	0.56	3.48	3.70	3.59	2	2
	20	3	0.27	0.38	0.33	3.40	3.77	3.61	3	3
Cefalexin (Vetranal)	80	2	2.07	2.12	2.10	2.39	2.55	2.47	0	0
	90	2	2.37	2.51	2.44	2.85	2.93	2.89	0	0
	100	4	2.44	2.74	2.57	3.15	3.61	3.31	0	0
	200	2	2.55	2.93	2.74	3.45	3.60	3.53	0	0

	500	2	1.76	1.91	1.84	3.54	3.79	3.67	0	0
	1000	20	0.65	1.24	0.90	2.87	3.81	3.37	17	17
	1250	20	0.40	0.84	0.59	2.30	4.52	3.04	20	20
	1500	4	0.17	0.22	0.21	2.72	2.97	2.84	4	4
	2000	4	0.07	0.15	0.12	3.17	3.44	3.31	4	4
	2500	4	0.01	0.04	0.02	2.39	4.22	3.30	4	4
Ceftiofur										
	5	3	1.27	1.43	1.33	2.17	2.31	2.25	0	0
	6	2	1.27	1.73	1.50	3.07	4.00	3.54	0	0
	7	2	1.31	1.45	1.38	3.30	3.70	3.5	0	0
	8	2	1.19	1.49	1.34	3.72	4.26	3.99	0	0
	9	20	0.31	1.28	0.93	2.49	4.57	3.48	16	16
	10	20	0.18	1.04	0.76	2.39	3.87	3.19	20	20
	15	2	0.31	0.35	0.33	2.78	2.91	2.85	2	2
Cefazolin										
	9	14	0.83	1.52	1.18	2.40	4.51	3.35	7	7
	10	20	0.81	1.49	1.09	2.41	4.06	3.16	11	11
	12	20	0.56	0.89	0.70	2.15	3.65	2.90	20	20
	16	4	0.32	0.34	0.33	4.39	4.81	4.68	4	4
	18	5	0.13	0.30	0.21	2.46	3.91	3.20	5	5
	20	4	0.03	0.27	0.13	3.76	4.63	4.18	4	4
Cephapirin										
	3	20	0.70	1.66	1.30	2.49	3.84	3.10	5	5
	4	20	0.30	0.99	0.80	2.27	3.43	2.74	20	20
	5	2	0.43	0.64	0.54	3.33	3.50	3.42	2	2
	6	2	0.31	0.38	0.35	2.98	3.17	3.08	2	2
	7	2	0.18	0.20	0.19	2.86	2.91	2.89	2	2
Cefoperazone										
	2	4	1.70	1.81	1.77	3.19	3.54	3.34	0	0
	3	20	0.62	1.65	1.17	2.02	3.92	3.13	9	9
	4	20	0.23	1.08	0.54	2.12	3.26	2.64	20	20
Cefacetil										
	10	5	0.51	1.35	1.05	2.86	3.28	3.03	2	2
	12	16	0.07	1.34	1.05	2.48	3.27	2.93	8	8
	14	20	0.54	1.40	1.05	2.22	3.61	3.02	14	14
	16	20	0.85	1.04	0.96	2.63	3.43	3.10	20	20
	20	2	0.46	0.53	0.50	3.12	3.21	3.17	2	2
	30	2	0.08	0.30	0.19	2.98	3.61	3.30	2	2
Cefalonium										
	2	2	1.27	1.72	1.50	2.40	2.95	2.68	0	0
	3	20	0.74	1.36	0.97	2.33	3.79	2.88	18	18
	4	20	0.48	1.01	0.73	2.39	4.51	3.29	20	20

Cefquinome	5	2	1.79	1.83	1.81	2.92	2.93	2.92	0	0
	10	3	0.86	1.88	1.33	3.05	4.22	3.44	1	1
	14	20	0.75	1.66	1.03	2.46	4.06	3.09	16	16
	16	20	0.57	1.04	0.79	2.44	3.86	3.17	20	20
	18	8	0.50	0.91	0.67	3.03	4.21	3.43	8	8
	20	2	0.30	0.39	0.35	3.17	3.26	3.22	2	2
	30	2	0.04	0.27	0.16	3.36	5.32	4.34	2	2
	40	2	0.01	0.07	0.04	3.53	4.07	3.80	2	2
Tetracycline	70	2	2.27	2.33	2.30	1.34	1.48	1.41	0	0
	80	20	1.97	2.82	2.46	0.78	1.40	1.10	11	11
	90	25	1.89	2.54	2.23	0.68	1.08	0.87	25	25
Chlortetracycline	40	2	2.53	3.10	2.82	1.36	1.77	1.57	0	0
	50	20	1.80	3.03	2.67	0.57	1.32	1.04	12	12
	60	20	1.86	3.21	2.57	0.49	1.02	0.74	20	20
	90	2	2.35	2.06	2.21	0.10	0.15	0.13	2	2
	100	2	2.32	2.61	2.47	0.01	0.12	0.07	2	2
	125	2	2.43	2.51	2.47	0.02	0.07	0.05	2	2
Oxytetracycline	60	4	2.17	2.46	2.33	1.08	1.52	1.28	2	2
	70	20	2.37	3.23	2.68	0.94	1.35	1.16	8	8
	80	20	2.09	3.24	2.72	0.70	1.18	0.92	17	17
	90	20	2.05	3.69	2.79	0.43	0.96	0.68	20	20
Doxycycline	40	2	2.03	2.95	2.49	0.86	1.54	1.25	5	5
	50	14	2.24	2.82	2.53	0.70	1.04	0.89	20	20
	60	22	2.53	2.58	2.56	0.45	0.52	0.49	2	2
	70	20	2.48	2.95	2.72	0.14	0.19	0.17	2	2