Validation report AnticFast® Beta-lactams & Tetracyclines Combo Test Kit

(Order n°: JC0084)

(Meizheng Bio-Tech, China)

June 15, 2021
Updated report of March 1, 2022

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1. Introduction

AnticFast® Beta-lactams & Tetracyclines Combo Test Kit (Meizheng Bio-Tech, China) is a qualitative two-step (2 min + 5 min) rapid lateral flow assay to detect β-lactam (penicillins and cefalosporins) and tetracycline antibiotic residues in raw commingled cows' milk.

A validation study was performed at ILVO-T&V (Technology & Food Science Unit of the Flanders research institute for agriculture, fisheries and food, Melle, BE) according to Commission Decision 2002/657/EC and to the CRL guidelines for the validation of screening methods for residues of veterinary medicines (*Anonymous*, 2010).
The following analytical parameters were checked: test specificity, detection capability, and test robustness (impact of deviation of the test protocol, impact of the milk composition or milk type, and batch differences of reagents). The test was also be included in the inter-laboratory study organized by ILVO in spring 2021.

Update of the report: determination of the detection capacity for ceftiofur with improved reagents (lot 3: 20210921G (expiration date 21/09/2022) and lot 4: 20211008G (expiration date 08/10/22)). Also 50 extra blank farm and 50 blank tanker milk samples were analysed using reagents of lot 3 and lot 4. All other validation (initial report) was performed using reagents lot 1: 20200826G (expiration date 26/08/2021) and lot 2: 20200812G (expiration date 12/08/2021).

2. Test procedure

Test preparation
All reagents and kit components should be at room temperature (20-25°C) before use. Ensure that the milk is homogenous (no precipitation no clotting). The milk samples should be at room temperature before testing. In this validation study, milk temperature was standard 20°C.
Remove the cover of the tube and take out the appropriate number of test strips and microwell. Immediately cover the tube and restore the remaining components at 2-8°C.

Test procedure
1st step: Transfer 200 µL of raw milk to each microwell placed in the incubator. Dissolve the coating conjugate in the microwell by pipetting the content up and down for 5 to 6 times.
2nd step: Incubate the sample for 2 minutes at room temperature (20-25°C), then insert the test strip into the microwell.
3rd step: Let the test strip develop color for 5 minutes at room temperature (20-25°C).
4th step: Take out the strip and remove the absorbent pad. Interpret the results within 1 minute visually or by using a BMZ6000 Portable Strip Reader and software.
For the test lines (T1 and T2) following counts: Negative: if the test line is stronger than or equal to the control line, the milk sample contains no antibiotics or contains antibiotics at lower level than the detection limits. Positive: test line is weaker (less intense) than the control line, the milk sample contains antibiotics above or equal to the detection limits.

2.1 Configuration of the AnticFast® Beta-lactams & Tetracyclines Combo Test Kit test strip

The configuration of the AnticFast® Beta-lactams & Tetracyclines Combo Test Kit is shown in Figure 1.

→ 'CTRL' line (control line)

→ 2
→ 1

2: test line for Tetracyclines
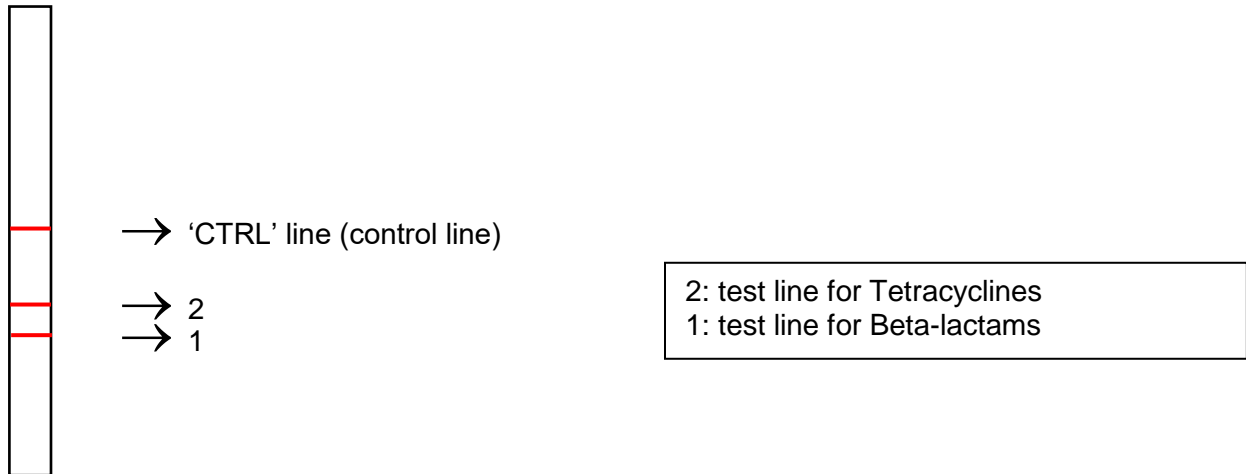1: test line for Beta-lactams

Fig. 1. Configuration of an AnticFast® Beta-lactams & Tetracyclines Combo Test Kit test strip.

2.2. Instrumental interpretation of the test

The BMZ6000 Portable Strip Reader is comparing the intensity of each test line with the intensity of the control (reference) line and calculates for each channel a ratio = intensity test line / intensity control line. This ratio for each test line is compared to a fixed cut-off value (ratio = 1.00).
The ratio cut-off levels are given in Table 1.
In order to perform an instrumental interpretation of a strip, the QR code, included for each channel in the test kit, needs to be scanned by the reader.

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

Ratio	Interpretation	Ratio	Interpretation
R≥1.00	negative	R<1.00	positive

Note: R: ratio.

Fig. 2. BMZ6000 Portable Strip Reader for instrumental reading of the color formation on the AnticFast® Beta-lactams & Tetracyclines Combo Test Kit.

2.3 Visual interpretation of the test

The kit manufacturer claims that visual reading of the AnticFast® Beta-lactams & Tetracyclines Combo Test Kit is possible. The intensity of the test lines is compared to the intensity of the reference (i.e. control) line. Negative: If the test line is stronger than or equal to the control line, the milk sample contains no antibiotics or contains antibiotics at lower level than the detection limits . Positive: test line is weaker (less intense) than the control line, the milk sample contains antibiotics above or equal to the detection limits. The interpretation is shown in Figure 3. Visual reading was not checked in this validation study.
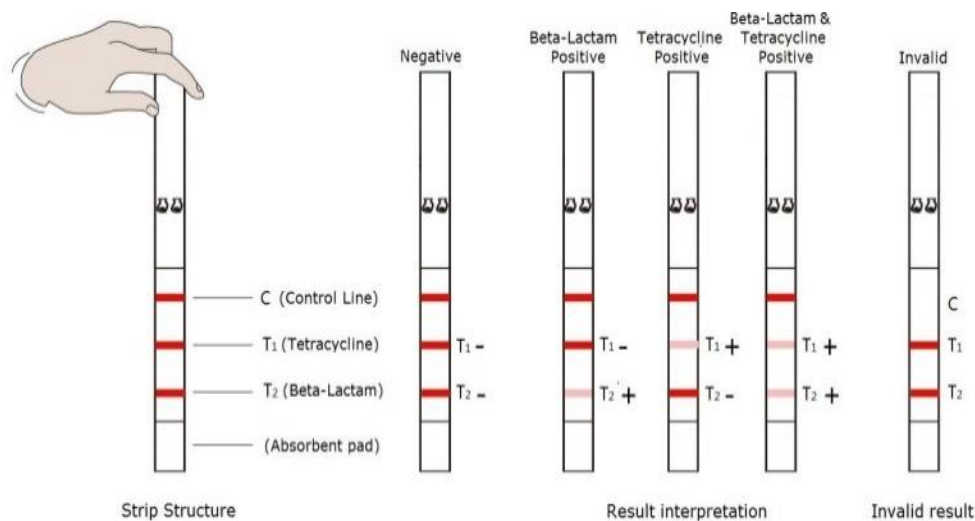

Fig. 3. Visual interpretation of the color formation on the AnticFast® Beta-lactams & Tetracyclines Combo Test Kit (*Anon.*; 2020).

3. Detection capability

Methods and Materials:

Spiking of antibiotic-free (blank) raw milk with β-lactams (penicillins and cefalosporins) and tetracyclines.
Blank milk was collected from 4 individual cows in mid-lactation which had not been treated with any veterinary drug for the last 2 months and which had a low to moderate number of somatic cells in the milk. Collected in sterile containers and kept below 4°C to limit the bacterial count. The maximum period for the cold storage of the fresh raw milk was 56 hours which is shorter than the local milk collection interval (3 days in Belgium).

The detection capability of the AnticFast® Beta-lactams & Tetracyclines Combo Test Kit was determined for all different compounds belonging to the β-lactam and tetracycline family mentioned as marker residue in Table 1 of the annex of Commission Regulation (EU) No 37/2010. The spiking was performed as described in the ISO Draft Technical Specification 23758 (ISO/IDF, 2020). Each compound was individually spiked in blank raw milk at fixed concentrations. For each compound a minimum of 2 concentrations around the test sensitivity (test detection capability) were tested. The increment between the concentrations tested for each compound was dependent on the level of spiking and the closeness to the respective MRL (Table 2).

Each concentration was tested 20, 40 or 60 times in a time period of at least three days.
o Tested concentration ≤0.5 MRL: 20 times
o Tested concentration >0.5 – <0.9 MRL: 40 times
o Tested concentration ≥0.9 – ≤1.0 MRL: 60 times
o Tested concentration >MRL: 20 times

Table 2. Increment between the concentrations tested for each compound was dependent on the level of spiking.

Concentration (in µg/kg)	Increment (in µg/kg)
1-10	1
11-20	2
21-50	5
51-100	10
101-250	25
251-500	50
501-1,000	100
1,001-5,000	500

The detection capability is defined as the lowest concentration tested where at least 19 out of 20 tests, 38 out of 40 tests or 57 out of 60 tests were positive, respectively.

Every day the following standards were also tested:

- blank raw milk free from antimicrobials - twice
- blank raw milk spiked with benzylpenicillin at 1 µg/kg and oxytetracycline at 3 µg/kg - twice
- blank raw milk spiked with cefalonium at 1 µg/kg - twice

Detection capability tests were performed with 4 different lots of reagents: lot 1 20200826G (expiration date 26/08/2021) and lot 2 20200812G (expiration date 12/08/2021), and for the detection capability of ceftiofur, two additional (more sensitive) lots were used: lot 3 20210921G (expiration date 21/09/2022) and lot 4 20211008G (expiration date 08/10/22). All were used following the manufacturer's instructions. The intensity of color formation of each test line was compared to the intensity of the control line and was interpreted by means of a BMZ6000 Portable Strip Reader and software. The cut-off value is 1.00 (≥1.00: negative; <1.00: positive). All results (reader values) were collected in a data base.

Table 3. Standard material used in this validations study.

Compound	Origin	Product number	Lot number
4-epimer of chlortetracycline	Acros Organics	268231000	A0406408
4-epimer of oxytetracycline	Acros Organics	257711000	A0395560
4-epimer of tetracycline	Acros Organics	233121000	A0397675
Amoxicillin trihydrate	Sigma Aldrich	31586	BCCB1309
Ampicillin trihydrate	Sigma Aldrich	31591	BCBS3642V
Cefacetrile	Sigma Aldrich	C231500	2-MAX-147-3
Cefalexin	Sigma-Aldrich	33989-100MG-R	lot BCBW7031
Cefalonium hydrate	Sigma Aldrich	32904	BCBV1595
Cefapirin sodium	Sigma Aldrich	43989	BCCC5208 BCBV1345
Cefazolin European Pharmacopeia reference standard	Sigma Aldrich	C0682800	5.0
Cefoperazone dihydrate	Sigma Aldrich	32426	BCBX0019
Cefquinome sulfate	Sigma Aldrich	32472	BCBW2550
Ceftiofur	Dr Ehrenstorfer	DRE-C11065000	G1104213
Chloramphenicol VETRANAL	Sigma Aldrich	31667	BCBR6685V
Chlortetracycline hydrochloride	Sigma Aldrich	46133	BCBT9837
Clavulanic acid (Potassium clavulante)	Sigma Aldrich	33454	STBJ0056
Cloxacilline sodium salt monohydrate	Sigma Aldrich	C9393	016M4853V
Colistin sulfate	Sigma Aldrich	C4461	049M4836V
Dapsone	Sigma Aldrich	46158	BCBX0187
Desacetylcephapirin	LGC Standards	682120	799058
Desfuroylceftiofur	TRC	D289980	5-WBZ-57-5
Dicloxacilline	Sigma Aldrich	46182	BCBX4662
Doxycycline Hyclate VETRANAL	Sigma Aldrich	33429	BCBS7684V
Enrofloxacin	Sigma Aldrich	33699	BCBZ6597
Erythromycin A dihydrate	Sigma Aldrich	46256	BCBS7769V
Lincomycin Hydrocloride Monohydrate	Sigma Aldrich	31727	BCBW4661
Nafcillin sodium salt VETRANAL	Sigma Aldrich	32071	BCCC5791
Neomycin trisulfate salt hydrate	Sigma Aldrich	N1876	SLBV5397
Oxacillin sodium salt monohydrate	Sigma Aldrich	46589	BCBT8512
Oxytetracycline Hydrochloride	Sigma Aldrich	O5875	057M4020V

Pencillin G sodium salt	Sigma Aldrich	PENNA	045M4815V
Sulfadiazine	Sigma Aldrich	S8626	056M4795V
Tetracycline hydrochloride	Sigma Aldrich	31741	BCCC9767
Trimethoprim, minimum 98%TLC	Sigma Aldrich	46984	BCBX8831

Certified reference material from following different reagent suppliers was used: Acros Organics (Geel, BE), Sigma-Aldrich N.V. (Overijse, BE), Toronto Research Chemicals (TRC) (Ontario, CA), Dr Ehrenstorfer (Augsburg, DE) and LGC Standards (Molsheim, FR). Detailed information of all standard material is given in Table 3.

Results:

A summary of the AnticFast® Beta-lactams & Tetracyclines Combo Test Kit detection capabilities is given in Table 4.

Table 4. Detection capability (in µg/kg) of AnticFast® Beta-lactams & Tetracyclines Combo Test Kit (Meizheng Bio-Tech, Beijing, CN) in raw bovine milk with instrumental reading (BMZ6000 Portable Strip Reader) with cut-off ratio = 1.00. Detection capability defined as the lowest concentration tested giving minimum 19, 38 or 57 positive results out of 20, 40 or 60 replicates, respectively.

Antibiotic Group/ antibiotic	EU MRL (µg/kg)	Detection capability (µg/kg)	
		Lot 1 & 2	Lot 3 & 4
Penicillins			
benzylpenicillin	4	1	
ampicillin	4	4	
amoxicillin	4	4	
oxacillin	30	4	
cloxacillin	30	3	
dicloxacillin	30	3	
nafcillin	30	18	
Cefalosporins			
ceftiofur	100[a]		80
desfuroylceftiofur	100[a]	**-- (>MRL) ***	
cefquinome	20	18	
cefazolin	50	50	
cephapirin	60[b]	16	
desacetylcephapirin	60[b]	40	
cefacetrile	125	35	
cefoperazone	50	3	
cefalexin	100	**-- (>MRL) ***	
cefalonium	20	1	

Table 4 continued

Antibiotic Group/ antibiotic	EU MRL (µg/kg)	Detection capability (µg/kg)	
		Lot 1 & 2	Lot 3 & 4
Tetracyclines			
tetracycline	100[c]	5	
4-epimer of tetracycline	100	5	
oxytetracycline	100[c]	2	
4-epimer of oxytetracycline	100	6	
chlortetracycline	100[c]	6	
4-epimer of chlortetracycline	100	9	
doxycycline	100[d]	2	

Notes: lot 1 20200826G (expiration date 26/08/2021), lot 2 20200812G (expiration date 12/08/2021), lot 3 20210921G (expiration date 21/09/2022) and lot 4 20211008G (expiration date 08/10/22).

*: detection capability >MRL, exact detection capability not determined. Bold and red font detection capabilities are above the drug MRL. MRL: Maximum Residue Limit, Regulation (EC) No 470/2009 and Commission Regulation (EU) No 37/2010 and amendments (situation on 01/02/2021). Detection capability defined as the lowest concentration tested giving a minimum of 19 positive results out of 20, 38 positive results out of 40 or 57 positive results out of 60, respectively.

[a]: The MRL of 100 µg/kg is applied on the sum of all residues retaining the β-lactam structure expressed as desfuroylceftiofur,

[b]: The MRL of 60 µg/kg in milk is applied on the sum of cephapirin and desacetylcephapirin,

[c]: The MRL of 100 µg/kg in milk is applied on the sum of parent drug and its 4-epimer,

[d]: No MRL in milk, not for use in animals from which milk is produced for human consumption.

Discussion:

The AnticFast® Beta-lactams & Tetracyclines Combo Test Kit is capable to detect the residues of β-lactams (penicillins and cefalosporins) and tetracyclines present on the EU-MRL list in milk (Commission Regulation (EU) No 37/2010) as included in Table 4. With the improved reagents, all β-lactams can be detected at least in 95% of the replicates at their respective MRL except for desfuorylceftiofur (CCβ>100 µg/kg), and cefalexin (CCβ>100 µg/kg). The exact detection capability for the two last antibiotics was not determined.

All tetracyclines (parent drugs and 4-epimers) can be detected at least in 95% of the replicates at their respective MRL. Doxycycline, not for use in animals from which milk is produced for human consumption, can be detected at least in 95% of the replicates from 2 µg/kg on. It is worth noted that all tetracyclines are detected below 10 µg/kg, the Russian milk standards (Customs Union, 2013) for tetracyclines in milk.

The test is fulfilling the current acceptance criteria (valid until 30 June 2023) and could be approved by the Belgian Federal Agency for the Safety of the Food Chain (FASFC) as test

used by the Belgian dairy companies to check incoming milk on the presence of β-lactam residues (*Anon.*, 2021).

4. Test selectivity and rate of false positive results
4.1. Test selectivity

Methods and Materials:
The selectivity of the different test lines of the AnticFast® Beta-lactams & Tetracyclines Combo Test Kit was tested by analysing milk spiked with β-lactam and tetracycline compounds and by analysing milk spiked with compounds belonging to different antibiotic or chemotherapeutic families (1 per family) to check the selectivity of the β-lactam test line. Raw milk was spiked at a high concentration (100×MRL or 100×MRPL in milk) in raw milk. All testing was completed in duplicate. In case of a positive result also lower concentrations were tested.

Following compounds were used: benzylpenicillin (penicillins), cefalonium (cefalosporins), oxytetracycline (tetracyclines), sulfadiazine (sulfonamides), neomycin B (aminoglycosides), erythromycin (macrolides), enrofloxacin (quinolones), chloramphenicol (amphenicols), colistin (polymyxins), lincomycin (lincosamides), clavulanic acid (β-lactamase inhibitors), trimethoprim (diamino pyrimidine derivatives) and dapsone (others chemotherapeutics).
Standard material from Sigma-Aldrich was used (Table 3).

Results:
A summary of the test selectivity is given in Table 5.

Discussion:
Clavulanic acid, a β-lactamase inhibitor, gave an interference at the beta-lactam channel. This interference is expected since this molecule contains a β-lactam structure resembling that of the penicillin, except that the fused thiazolidine ring of the penicillins is replaced by an oxazolidine ring (*Anon.*, 2005). Interference by clavulanic acid with a 95% or higher detection was from 1,500 µg/kg on.

AnticFast® Beta-lactams & Tetracyclines Combo Test Kit is a highly specific test for detection of β-lactams in milk and does not detect compounds from the tetracyclines, sulfonamides, aminoglycosides, macrolides, quinolones, amphenicols, polymyxins, lincosamides and diamino pyrimidine derivatives, nor dapsone.

Table 5. Ratios obtained for compounds of different antibiotic families spiked in raw milk and tested with AnticFast® Beta-lactams & Tetracyclines Combo Test Kit

Family	Compound	MR(P)L (µg/kg)	Conc. spiked in milk (µg/kg)	BL channel		Tet channel	
				Ratio	Result	Ratio	Result
Penicillins	Benzylpenicillin	4	400	0.2677	+	1.8152	-
Cefalosporins	Cefalonium	20	2,000	0.2236	+	1.7696	-
Tetracyclines	Oxytetracycline	100[a]	10,000	1.6844	-	0.1506	+
Sulfonamides	Sulfadiazine	100[b]	10,000	1.9613	-	1.7856	-
Aminoglycosides	Neomycin B	1,500	150,000	1.5812	-	1.5689	-
Macrolides	Erythromycin	40	4,000	1.8510	-	1.7594	-
Quinolones	Enrofloxacin	100[c]	10,000	2.0614	-	1.8339	-
Amphenicols	Chloramphenicol	0.3[d]	30	2.1652	-	1.9851	-
Polymyxins	Colistin	50	5,000	2.1429	-	1.9468	-
Lincosamides	Lincomycin	150	15,000	2.1570	-	1.9435	-
β-lactamase inhibitors	Clavulanic acid	200	20,000	**0.3676**	**+**	1.8453	-
Diamino pyrimidine derivatives	Trimethoprim	50	5,000	1.9747	-	1.7943	-
Others	Dapsone	5[e]	500	1.9104	-	1.7010	-

Notes: MRL: Maximum Residue Limit, Regulation (EC) No 470/2009 and Commission Regulation (EU) No 37/2010 and amendments (situation on 01/02/2021).Conc.: concentration; BL: β-lactam; Tet: tetracycline.

[a]: The MRL of 100 µg/kg in milk is applied on the sum of parent drug and its 4-epimer;

[b]: The combined total residues of all substances within the sulphonamide group should not exceed 100 µg/kg

[c]: The MRL of 100 µg/kg in milk is applied on the sum of enrofloxacin and ciprofloxacin;

[d]: Prohibited substance, MRPL (Minimum Required Performance Limit, Commission Decision (EC) No 2003/181/EC);

[e]: Prohibited substance, Recommended concentration for testing (*Anon.*, 2007).

4.2. Test for false-positive/false-negative results

Methods and materials:
305 blank farm and 301 tanker load milk samples were tested with lot 1 and 2 of AnticFast® Beta-lactams & Tetracyclines Combo Test Kit and other microbiological and receptor screening tests. With lot 3 and 4 (improved reagents), 50 additional blank farm and 50 tanker load milk samples were tested, as it was noticed that the ratios obtained for blank samples were lower than with lot 1 and 2.

Results and discussion:
Lot 1 and 2: Of the 305 farm milk samples, 300 tested negative for β-lactams and tetracyclines on AnticFast® Beta-lactams & Tetracyclines Combo Test Kit. Five farm milk samples tested positive on the tetracycline channel, but were proven to be real positives by use of other screening tests. LC-MS/MS analysis of one of these positive samples indicated the presence of 3.2 µg/kg of oxytetracycline.

Table 6a. AnticFast® Beta-lactams & Tetracyclines Combo Test Kit results for blank farm and tanker milk samples.

Lot 1 and 2	Farm milk (n=300)		Tanker milk (n=299)	
	Ratio		Ratio	
	BL	TET	BL	TET
Mean	1.8210	1.6445	1.9240	1.6954
Min	1.3946	1.0990	1.6376	1.4315
Max	2.4089	2.0699	10.6149	9.3904
s_r	0.15	0.12	0.52	0.46
CV%	8.00	7.00	26.87	26.88

Notes: mean: mean ratio; min: lowest ratio; max: highest ratio; BL: β-lactam and TET: tetracycline. s_r ; Standard Deviation; CV(%): Relative Standard Deviation.

Of the 301 tanker load milk samples all tested negative on the tetracycline channel. On the beta-lactam channel two samples of the tanker load milk samples tested positive of which one was proven to be a real positive by other screening tests. Repetition *in duplo* of the other positive tested sample (ratio 0.9281) gave another positive (ratio 0.9850) and one negative (ratio: 1.0171) result on AnticFast® Beta-lactams & Tetracyclines Combo Test Kit. Other screening tests gave negative results, but were not able to detect all Beta-lactams at such a low levels. The sample gave borderline results and with LC-MS/MS analysis, a very small concentration of 0.55 µg/kg of cefalonium was found (CCβ for cefalonium on AnticFast® Beta-lactams & Tetracyclines Combo Test Kit = 1 µg/kg).

Giving these results, it is concluded that in total no false positive results were obtained upon 599 samples on both channels. The results for the real blank milk samples are summarized in Table 6a.

Table 6b. AnticFast® Beta-lactams & Tetracyclines Combo Test Kit results for blank farm and tanker milk samples.

Lot 3 and 4	Farm milk (n=50)		Tanker milk (n=50)	
	Ratio		Ratio	
	BL	TET	BL	TET
Mean	1.4294	1.7046	1.4238	1.6894
Min	1.2844	1.5836	1.2891	1.5407
Max	7.9987	6.9999	1.5328	1.8606
s_r	0.08	0.07	0.04	0.06
CV%	5.86	4.12	3.11	3.27

Notes: mean: mean ratio; min: lowest ratio; max: highest ratio; BL: β-lactam and TET: tetracycline. s_r ; Standard Deviation; CV(%): Relative Standard Deviation.

For the improved reagents of lot 3 and 4: all 50 blank farm milk and all 50 tanker samples, tested negative for β-lactams and tetracyclines on AnticFast® Beta-lactams & Tetracyclines

Combo Test Kit. Giving these results, it is concluded that in total no false positive results were obtained upon 100 samples on both test channels with the improved reagents. The results are summarized in Table 6b.

5. Reader and test repeatability
5.1 Repeatability of the reader

Methods and Materials:
Ten blank,10 low positive samples and 10 high positive samples for each channel were tested and the dipstick measured twice.

Results:
The results of the repeatability of the reader on the AnticFast® Beta-lactams & Tetracyclines Combo Test Kit results are summarized in Table 7. For the spiked milk only the relevant data for the different channels are presented.

Discussion:
The repeatability of the reader was very good; very low standard deviations of repeatability were obtained (relative standard deviations: highest value on β-lactam channel: 1.23%; highest value on tetracycline channel 0.94%).

Table 7. Repeatability of the reader

Reader repeatability	Beta-lactam			Tetracyclines		
	Mean ratio	s_r	CV%	Mean ratio	s_r	CV%
Blank milk	1.8356	0.01	0.51	1.7057	0.02	0.94
Low positive milk	0.8443	0.01	1.23	0.9101	0.00	0.14
High positive milk	0.4417	0.00	0.50	0.3923	0.00	0.59

Notes: s_r: Standard deviation of repeatability; CV(%): Relative standard deviation.

5.2 Repeatability of the test

Methods and Materials:
Ten blank,10 low positive samples and 10 high positive samples for each channel were analysed twice.

Results:
The results of the repeatability of the AnticFast® Beta-lactams & Tetracyclines Combo Test Kit are summarized in Table 8. For the spiked milk only the relevant data for the different channels are presented.

Table 8: Repeatability of the test

Test repeatability	Beta-lactam			Tetracyclines		
	Mean ratio	s_r	CV%	Mean ratio	s_r	CV%
Blank milk	1.7426	0.07	4.28	1.6003	0.06	3.99
Low positive milk	0.8797	0.03	2.98	0.3777	0.03	7.61
High positive milk	0.5136	0.02	3.65	0.8440	0.04	4.55

Notes: s_r: Standard deviation of repeatability; CV(%): Relative standard deviation.

Discussion:

The repeatability of the test was also very good, low standard deviation values were obtained. The highest variance value on β-lactam channel: 4.28%; highest variance value on tetracycline channel 7.61%.

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6. Test robustness

6.1. Influence of changes in the test protocol on the test results

In order to determine the robustness of the assay, the timing of the steps, a delay in reading or the milk volume in the protocol was changed.

6.1.1. Influence of the length of the incubation steps on the test results

In order to determine the robustness of the assay, the timing of the incubation steps in the protocol was changed. The normal incubation takes 2+5 minutes.

Methods and Materials:

Blank and spiked milk samples containing benzylpenicillin at 1 µg/kg and tetracycline at 3 µg/kg or spiked milk containing cefalonium at 1 µg/kg were analysed (3 replicates) with a test protocol with incubation timings different from the test protocol (reference = 2'+5').

Results:

The results of the influence of the length of the incubation steps on the AnticFast® Beta-lactams & Tetracyclines Combo Test Kit results are summarized in Table 9.

Discussion:

Most variations in the length of the incubation steps did not impact results significantly; all negative results remained negative and nearly all positive results stayed positive. Milk spiked with cefalonium at 1 µg/kg did show slightly higher ratios, and resulted in a borderline negative results (ratio: 1.0143) when the second incubation time was 30 seconds less than recommended. So it is recommended to stick to the timings as set in the kit insert.

Table 9. Impact of the length of the incubation steps on the AnticFast® Beta-lactams & Tetracyclines Combo Test Kit results (ratio).

Length of incubation steps	Ratio					
	Blank milk		Spiked milk with benzylpenicillin at 1 µg/kg & oxytetracycline at 3 µg/kg		Spiked milk with cefalonium at 1 µg/kg	
	BL	TET	BL	TET	BL	TET
1 minutes 45 seconds + 5 minutes 30 seconds						
mean	1.8497	1.6461	0.5943	0.6859	0.6868	1.5692
min	1.7767	1.5901	0.5370	0.6385	0.5590	1.5112
max	1.9656	1.7285	0.6825	0.7568	0.8386	1.6268
1 minutes 45 seconds + 5 minutes						
mean	1.8713	1.6588	0.6607	0.7204	0.8292	1.6875
min	1.7046	1.5656	0.5899	0.6575	0.6892	1.6110
max	2.0219	1.7830	0.7549	0.7945	0.9745	1.7749
1 minutes 45 seconds + 4 minutes 30 seconds						
mean	1.7631	1.6221	0.6449	0.6853	0.8133	1.6395
min	1.5660	1.5960	0.6111	0.6345	0.6267	1.5666
max	1.8620	1.6521	0.6990	0.7644	0.9568	1.7753
2 minutes + 4 minutes 30 seconds						
mean	2.0327	1.7369	0.6209	0.6913	0.8202	1.6958
min	1.8705	1.6579	0.5266	0.5849	0.6580	1.5557
max	2.2492	1.8613	0.7721	0.8361	**1.0143**	1.9645
2 minutes + 5 minutes (REF)						
mean	1.9923	1.7567	0.6697	0.6804	0.7368	1.6360
min	1.8319	1.6420	0.5842	0.6160	0.5915	1.5277
max	2.1342	1.8595	0.8361	0.8059	0.8651	1.7714
2 minutes + 5 minutes 30 seconds						
mean	1.8753	1.6671	0.6176	0.6818	0.6858	1.6153
min	1.7147	1.5881	0.4907	0.6281	0.5327	1.5308
max	1.9817	1.7521	0.7485	0.7145	0.7917	1.6879
2 minutes 15 seconds + 4 minutes 30 seconds						
mean	1.8822	1.6507	0.5435	0.6586	0.6917	1.6085
min	1.8017	1.6227	0.4455	0.5827	0.5514	1.5364
max	1.9823	1.6855	0.6176	0.7898	0.8286	1.7256
2 minutes 15 seconds + 5 minutes						
mean	1.9938	1.7639	0.6244	0.6991	0.7572	1.6870
min	1.8654	1.6409	0.5873	0.6070	0.5956	1.6288
max	2.1988	1.9133	0.6680	0.7993	0.9015	1.7753
2 minutes 15 seconds + 5 minutes 30 seconds						
mean	1.7772	1.5759	0.4570	0.6325	0.6726	1.5611
min	1.6193	1.4985	0.4044	0.5355	0.5301	1.5105
max	1.8965	1.6487	0.5177	0.7249	0.7996	1.6432

Notes: REF: reference; mean: mean ratio; min: minimum ratio; max: maximum ratio; BL: β-lactam; TET: Tetracycline.

6.1.2. Delay of reading

Methods and Materials:

Blank and spiked milk samples containing benzylpenicillin at 1 µg/kg and oxytetracycline at 3 µg/kg or spiked milk containing cefalonium at 1 µg/kg (3 replicates) were tested with a test protocol with a delay of the reading after the end of the incubation. A delay of 5 and 10 minutes was tested and the results compared with no delay in reading (= reference). The kit manufacturers advises to read the result within 1 minute.

Results:
The results of the influence of the delay of reading are summarized in Table 10.

Table 10. Ratios obtained when testing blank and spiked milk samples and reading the AnticFast® Beta-lactams & Tetracyclines Combo Test Kit strips directly after incubation or with a delay of 5 or 10 minutes respectively.

Ratio	Delay of reading					
	No delay (REF)		5 min		10 min	
	BL	TET	BL	TET	BL	TET
Blank milk						
mean	1.9923	1.7567	1.8713	1.7195	1.8327	1.7018
min	1.8319	1.6420	1.7046	1.6155	1.6970	1.5973
max	2.1342	1.8595	2.0219	1.8037	1.9480	1.7871
Milk spiked with benzylpenicillin at 1 µg/kg and oxytetracycline at 3 µg/kg						
mean	0.6697	0.6804	0.7882	0.7587	0.7066	0.7171
min	0.5842	0.6160	0.6974	0.6872	0.5613	0.6238
max	0.8361	0.8059	0.9100	0.8496	0.8433	0.8256
Milk spiked with cefalonium at 1 µg/kg						
mean	0.7368	1.6360	0.8316	1.5999	0.7583	1.6243
min	0.5915	1.5277	0.7558	1.5483	0.6108	1.5212
max	0.8651	1.7714	0.9035	1.6645	0.8811	1.7663

Notes: REF: reference; mean: mean ratio; min: minimum ratio; max: maximum ratio; BL: β-lactam; TET: Tetracycline.

Discussion:
Delay in reading the devices did not significantly impact the interpretation of test results: all negative results remained negative and all other positive results stayed positive.

6.1.3. Volume of the milk

Methods and Materials:
Blank and spiked milk samples containing benzylpenicillin at 1 µg/kg and oxytetracycline at 3 µg/kg or spiked milk containing cefalonium at 1 µg/kg (3 replicates) with a test protocol with different volumes of milk. A volume of 180, 200 (protocol = reference), and 220 µl of milk was tested.

Results:
The results of the influence of the different volumes of milk are summarized in Table 11.

Discussion:
A volume of milk differing some 20 µl (10%) from the prescribed volume of 200 µl did not impact the interpretation of test results; the negative results remained negative and positive results stayed positive. For the spiked milk, a milk volume of 220 µl gave slightly decreased ratios (become more positive).

Table 11. Ratios obtained when testing different volumes (180, 200 and 220 µl, respectively) of milk with with AnticFast® Beta-lactams & Tetracyclines Combo Test Kit.

Ratio	Volume of milk					
	180 µl		200 µl (REF)		220 µl	
	BL	TET	BL	TET	BL	TET
Blank milk						
mean	1.9110	1.7808	1.9923	1.7567	1.9593	1.7150
min	1.7737	1.6808	1.8319	1.6420	1.7720	1.5990
max	2.1372	1.8621	2.1342	1.8595	2.1998	1.8800
Milk spiked with benzylpenicillin at 1 µg/kg and oxytetracycline at 3 µg/kg						
mean	0.6178	0.6501	0.6697	0.6804	0.5420	0.6155
min	0.5495	0.5908	0.5842	0.6160	0.4866	0.5576
max	0.7520	0.7432	0.8361	0.8059	0.6181	0.6987
Milk spiked with cefalonium at 1 µg/kg						
mean	0.7010	1.6885	0.7368	1.6360	0.6938	1.6742
min	0.3147	1.5706	0.5915	1.5277	0.5593	1.6241
max	0.9671	1.8452	0.8651	1.7714	0.7880	1.7532

Notes: REF: reference; mean: mean ratio; min: minimum ratio; max: maximum ratio; BL: β-lactam; TET: Tetracycline.

6.2. External influences

6.2.1. Impact of the milk temperature

Methods and Materials:
Tests were performed (3 replicates) with milk of 20°C (= reference) and of 1-4°C in order to check if the milk temperature is influencing the AnticFast® Beta-lactams & Tetracyclines Combo Test Kit result. Besides blank milk also spiked milk samples containing benzylpenicillin at 1 µg/kg and oxytetracycline at 3 µg/kg or spiked milk containing cefalonium at 1 µg/kg were used.

Results:
The results of the impact of the milk temperature are summarized in Table 12.

Discussion:
The milk temperature (4°C) did not significantly impact the AnticFast® Beta-lactams & Tetracyclines Combo Test Kit results: blank milk was always tested as negative while the spiked milk samples gave a clear positive result on their respective channel. For milk at 4°C, slightly lower ratios were obtained and a smaller range of variation.

Table 12. Impact of the milk temperature on the AnticFast® Beta-lactams & Tetracyclines Combo Test Kit result.

	Milk temperature			
Sample	**1-4°C**		**20°C (REF)**	
	BL	**TET**	**BL**	**TET**
Blank milk				
mean	1.8964	1.6834	1.9923	1.7567
min	1.7687	1.6278	1.8319	1.6420
max	1.9705	1.7763	2.1342	1.8595
Milk spiked with benzylpenicillin at 1 µg/kg and oxytetracycline at 3 µg/kg				
mean	0.6174	0.6564	0.6697	0.6804
min	0.5801	0.6084	0.5842	0.6160
max	0.6537	0.7325	0.8361	0.8059
Milk spiked with cefalonium at 1 µg/kg				
mean	0.7271	1.6678	0.7368	1.6360
min	0.6612	1.6055	0.5915	1.5277
max	0.7910	1.7057	0.8651	1.7714

Notes: REF: reference; mean: mean ratio; min: minimum ratio; max: maximum ratio.

6.3. Milk composition and milk type influences

Methods and Materials:
The influence of milk compositional components or milk quality, low and high fat, low and high protein was compared with blank milk of normal quality /composition, and spiked near CCβ with benzylpenicillin at 1 µg/kg & oxytetracycline at 3 µg/kg, and cefalonium at 1 µg/kg. Ten replicates of each milk type were performed.

Fat content
Normal milk samples and milk samples with a low (<1.79 g per 100 ml) or high (> 6.01 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 AnticFast® Beta-lactams & Tetracyclines Combo Test Kit result.
The milk samples with a low fat content were natural milk samples with a low fat content selected at the milk control station based on infrared spectroscopic results with a MilcoScan 4000.

Protein content
Normal milk samples and milk samples with a low (<2.98g per 100 ml) or a high (>4.01 g per 100 ml) protein content were analysed and the ratios obtained were compared in order to study the impact of the protein content on the AnticFast® Beta-lactams & Tetracyclines Combo Test Kit result. The milk samples tested were natural milk samples with a low and a high protein content. These samples were selected at the milk control station based on infrared spectroscopic results with a MilcoScan 4000.

Results:
With respect to the impact of the milk composition (fat and protein content), the mean, the highest and lowest reader value are given in Figures 4 to 6 and Table 13 for blank milk, milk spiked with benzylpenicillin & oxytetracycline, and cefalonium, respectively.
.

The legend for the different situations in Figure 4 to 6.
 1 = Reference: normal raw milk;
 2 = Low fat content (<1.79 g/100 ml);
 3 = High fat content (>6.01 g/100 ml);
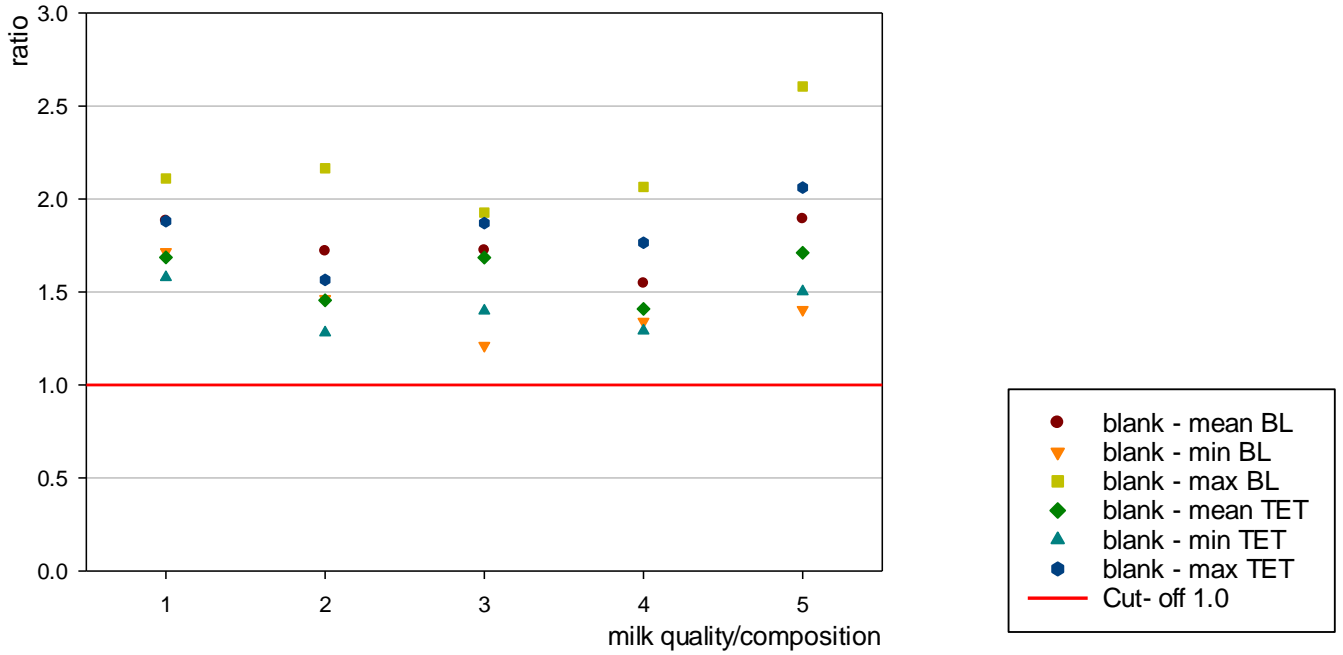 4 = Low protein (<2.98 g/100 ml);
 5 = High protein (>4.01 g/100 ml);

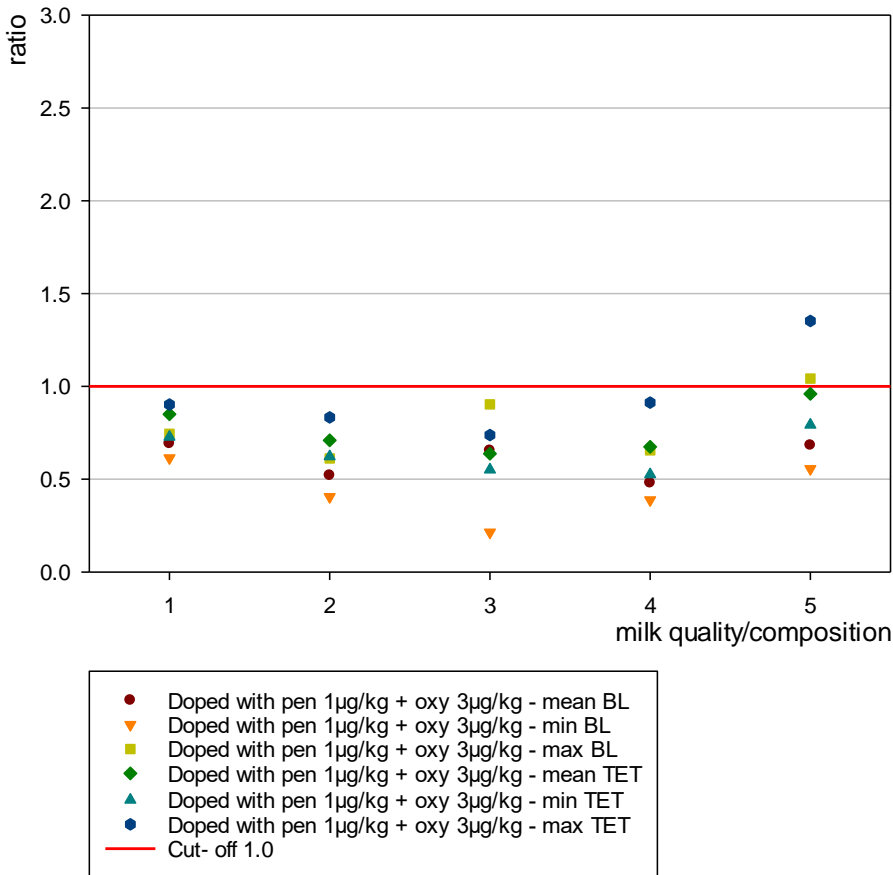Fig. 4. Results for blank milk, 10 replicates. BL: β-lactam channel; TET: Tetracycline channel.

Fig. 5. Results for milk spiked with benzylpenicillin at 1 µg/kg and oxytetracycline at 3 µg/kg, 10 replicates. BL: β-lactam channel; TET: Tetracycline channel.
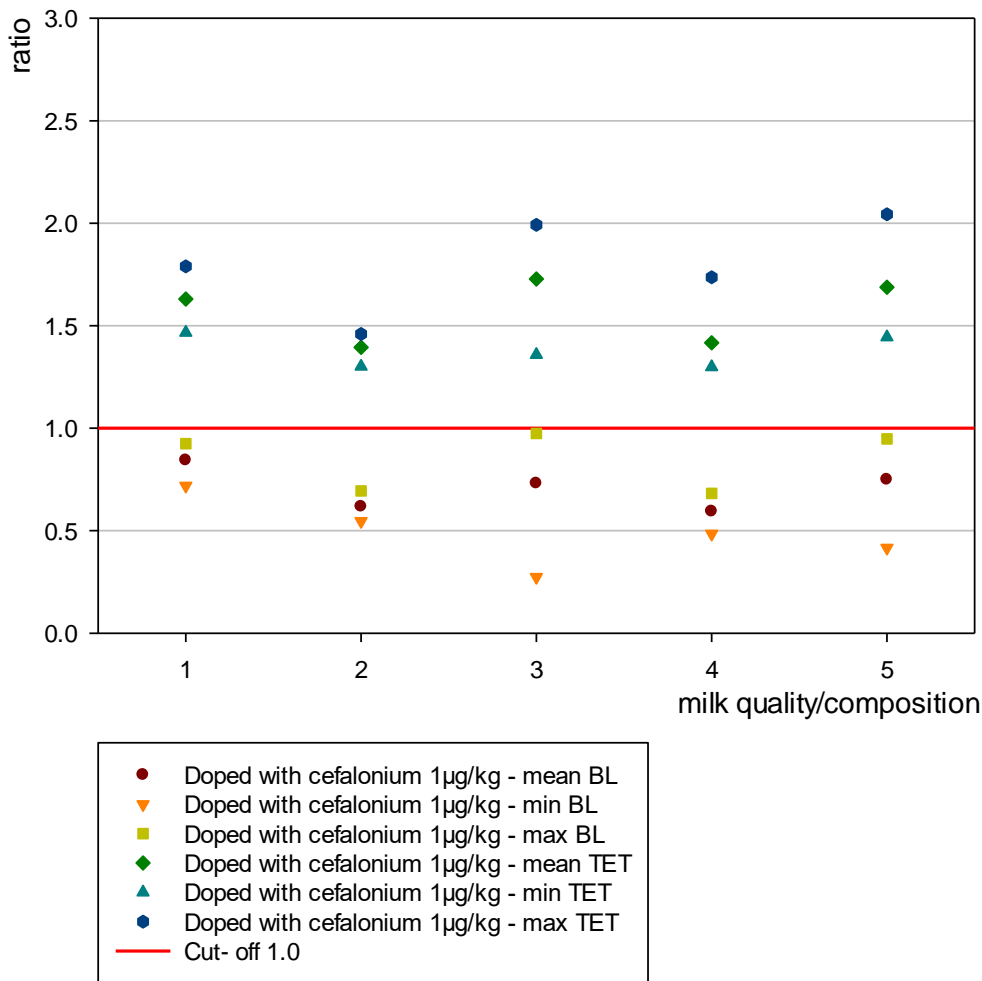
Fig. 6. Results for milk spiked with cefalonium at 1 µg/kg, 10 replicates. BL: β-lactam channel; TET: Tetracycline channel.

Discussion:

In general, the fat content and protein content had mostly no significant influence on the performance of the AnticFast® Beta-lactams & Tetracyclines Combo Test Kit result. No false positives were obtained with the blank milk. And all positive samples were positive except for milk with a high protein content spiked with benzylpenicillin at 1 µg/kg and oxytetracycline at 3 µg/kg, where on the β-lactam one borderline negative result (ratio 1.0415) and on the tetracycline channel three borderline (ratios between 1.0222 and 1.0906) and one negative (ratio 1.3529) results were obtained. This indicates a hampering of detection for high protein milk at these low concentrations. However, since the samples were spiked at concentrations far below the MRL (MRL of benzylpenicillin at 4 µg/kg and MRL of oxytetracycline at 100 µg/kg), no detection problems at MRL are expected for these compounds. But the results indicate that higher detection capabilities could be possible in milk with a high protein content which could be problematic for the detection of ampicillin or amoxicillin.

Table 13. AnticFast® Beta-lactams & Tetracyclines Combo Test Kit results for blank and spiked normal raw cows' milk and for blank and spiked milk of special composition (10 replicates).

	Ratio					
	Beta-lactam channel			Tetracycline channel		
	mean	min	max	mean	min	max
Blank raw cows' milk						
normal milk = reference	1.8827	1.7166	2.1104	1.6867	1.5789	1.881
low fat <1.79 g/100 g	1.7202	1.4646	2.1658	1.4558	1.2814	1.5652
high fat >6.01 g/100 g*	1.7245	1.2115	1.9272	1.6856	1.3993	1.8709
low protein <2.98 g/100 ml	1.5465	1.3418	2.0651	1.4097	1.2924	1.7652
high protein >4.01 g/100 ml	1.8934	1.4049	2.6051	1.7110	1.5027	2.0621
Milk with benzylpenicillin at 1 µg/kg and oxytetracycline at 3 µg/kg						
normal milk = reference	0.6915	0.6140	0.7447	0.8499	0.7271	0.9018
low fat <1.79 g/100 g	0.5200	0.4052	0.6118	0.7093	0.6217	0.8324
high fat >6.01 g/100 g*	0.6533	0.2138	0.9027	0.6370	0.5505	0.7380
low protein <2.98 g/100 ml	0.4798	0.3881	0.6556	0.6745	0.5249	0.9122
high protein >4.01 g/100 ml	0.6817	0.5560	**1.0415**	0.9598	0.7921	**1.3529**
Milk with cefalonium at 1 µg/kg						
normal milk = reference	0.8421	0.7185	0.9242	1.6303	1.4669	1.7905
low fat <1.79 g/100 g	0.6167	0.5462	0.6939	1.3948	1.3013	1.4604
high fat >6.01 g/100 g*	0.7302	0.2741	0.9740	1.7281	1.3594	1.9920
low protein <2.98 g/100 ml	0.5940	0.4843	0.6815	1.4175	1.2989	1.7368
high protein >4.01 g/100 ml	0.7483	0.4158	0.9476	1.6882	1.4449	2.0440

Notes: *: decreased flow possible; min: minimum; max: maximum.

6.4. Type of milk and animal origin influences

Methods and Materials:

This evaluation looked at the influence of different types of milks: UHT, goats' and ewes' milk. In this evaluation n=10 negative raw cows' milk, and n=10 negative UHT milk, goats' and ewes' milk each spiked with benzylpenicillin at 4 µg/kg, cefalonium at 5 µg/kg or oxytetracycline at 100 µg/kg were tested and evaluated. Minimum, maximum and mean are plotted in Figures 5 to 8 for blank, benzylpenicillin at 4 µg/kg, cefalonium at 5 µg/kg and oxytetracycline at 100 µg/kg, respectively.

Raw cows' milk, UHT milk, sterilized milk and reconstituted milk powder were analysed in order to determine if the AnticFast® Beta-lactams & Tetracyclines Combo Test Kit is a suitable test for these types of milk. Raw goats' milk and raw ewes' milk samples were analysed to determine if the AnticFast® Beta-lactams & Tetracyclines Combo Test Kit is a suitable test for these types of milk coming from animal species other than the cow. Each milk type was tested as such (blank) and spiked near CCβ with benzylpenicillin at 1 µg/kg & oxytetracycline at 3 µg/kg, and cefalonium at 1 µg/kg. Ten replicates of each milk type were performed.

Results:

With respect to the impact of the milk type (UHT, sterilized, reconstituted milk powder and thawed milk) and animal origin (goats' and ewes' milk), the mean, the highest, and lowest reader value for each milk type are given in Figures 7 to 9 and Table 14.

The legend for the different situations in Figures 7 to 9:

1 = Reference: normal raw milk;
2 = UHT milk;
3 = Sterilized milk;
4 = Reconstituted milk powder;
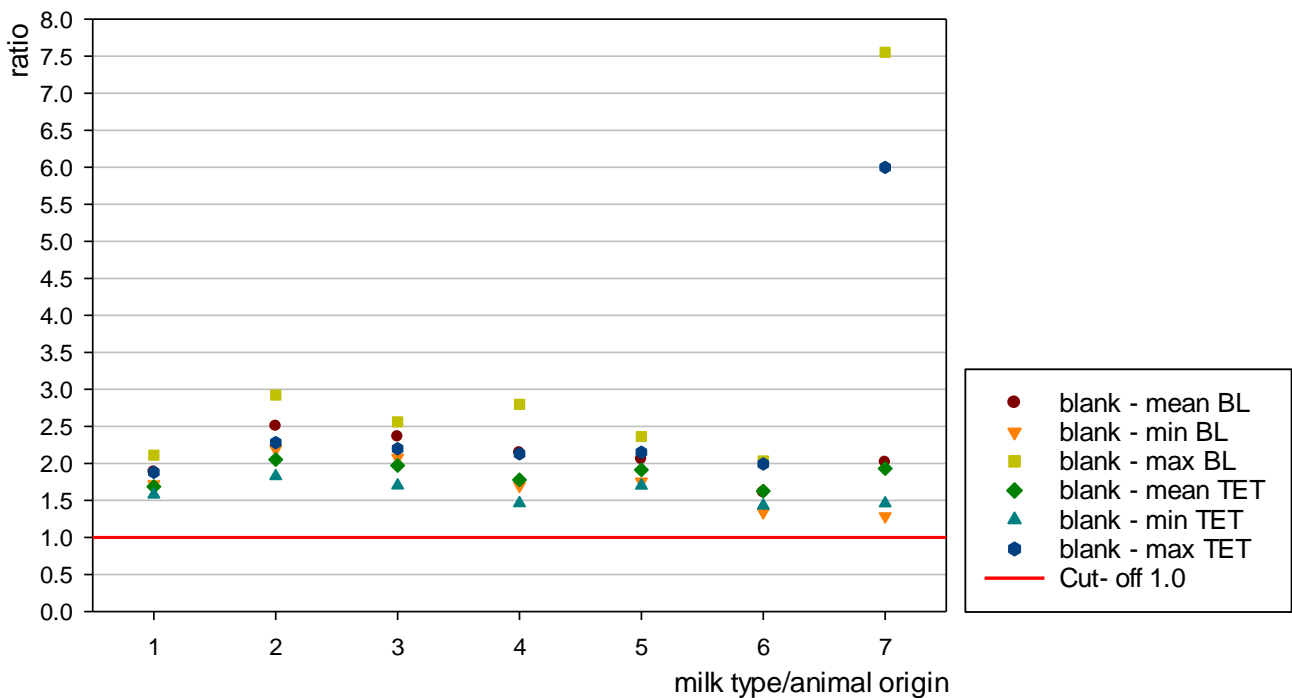5 = Thawed milk;
6 = Goats' milk;
7 = Ewes' milk

Fig. 7. Results for blank milk, 10 replicates. BL: β-lactam channel; TET: Tetracycline channel.
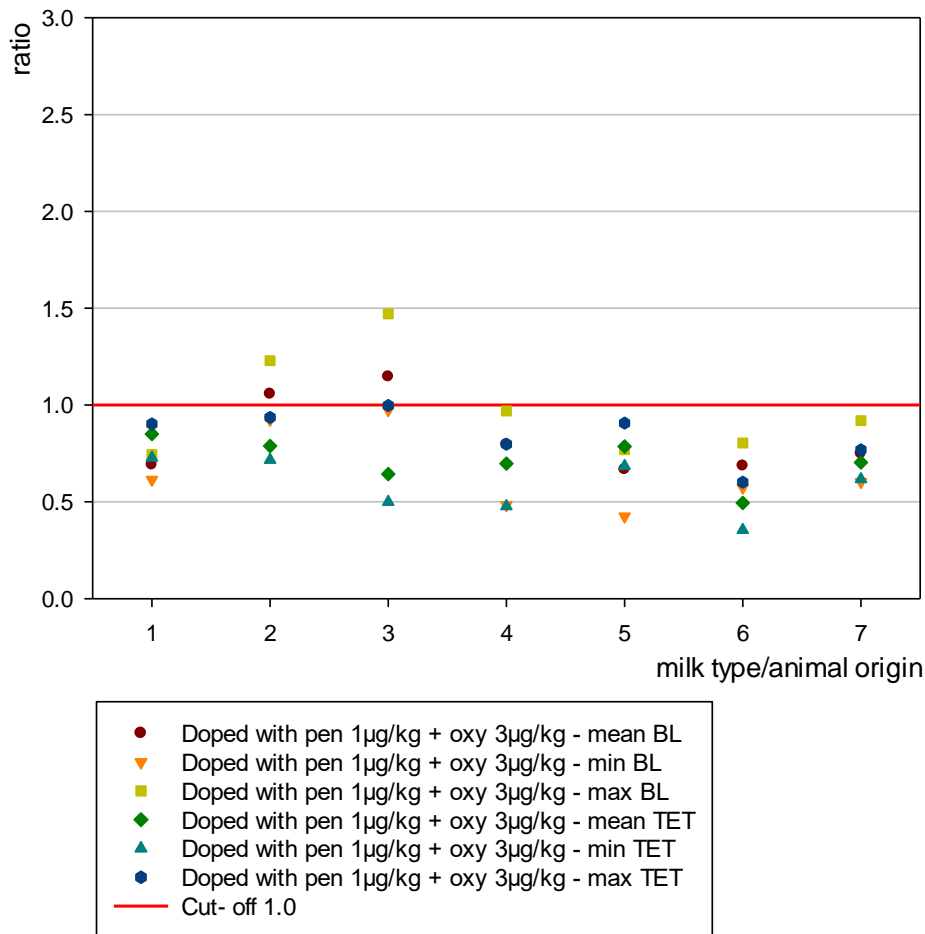
Fig. 8. Results for milk spiked with benzylpenicillin at 1 µg/kg and tetracycline at 3 µg/kg, 10 replicates. BL: β-lactam channel; TET: Tetracycline channel.

Discussion:

There could also be interest to use the AnticFast® Beta-lactams & Tetracyclines Combo Test Kit, although developed for the testing of raw cows' milk, to test UHT milk, sterilized milk, thawed milk (monitoring samples are often kept frozen during transport and storage) or reconstituted milk powder. One could also have interest to test milk from an animal species different from the cow (goat, ewe).

For all milk types other than raw cows' milk, a diminished flow was observed. Further, for blank milk, all types of milk gave negative results. For spiked milk types, UHT and sterilized milk samples obtained some negative results on the β-lactam channel. For UHT, 6 out of 10 negative results for milk spiked with benzylpenicillin at 1 µg/kg and oxytetracycline at 3 µg/kg; ratio range: 1.0397 - 1.2284) and 9 out of 10 negative results for milk spiked with cefalonium at 1 µg/kg; ratio range: 1.1215 - 1.5513) were obtained. Not only a diminished flow was observed for UHT milk, all test lines were also clearly lighter of color. All spiked sterilized milk samples gave in 9 out of 10 samples negative results on the β-lactam channel. And also for reconstituted milk powder, 4 out of 10 negative results were obtained when spiked with cefalonium at 1 µg/kg. Therefore it can be concluded that an impact on the detection occurs

with heat threated milk such as UHT, sterilized milk and reconstituted milk powder. So, there is no guarantee that in such milk types ampicillin and amoxicillin could always be detected at MRL. Also for spiked thawed milk 3 out of 10 results, negative results of which 2 borderline (ratio: 1.005 and 1.0449) were obtained when spiked with cefalonium at 1 µg/kg.

A diminished flow was also observed with blank ewes' milk, all blank milk remained negative, but for one sample the control line and tetracycline test line were not formed causing a very high ratio on both channels. For ewes' milk spiked with cefalonium at 1 µg/kg milk 2 negative results out of 10 were obtained (ratios: 1.005 and 1.1376). Blank goats' milk samples also always obtained negative results. Spiked goats' milk gave all positive results.
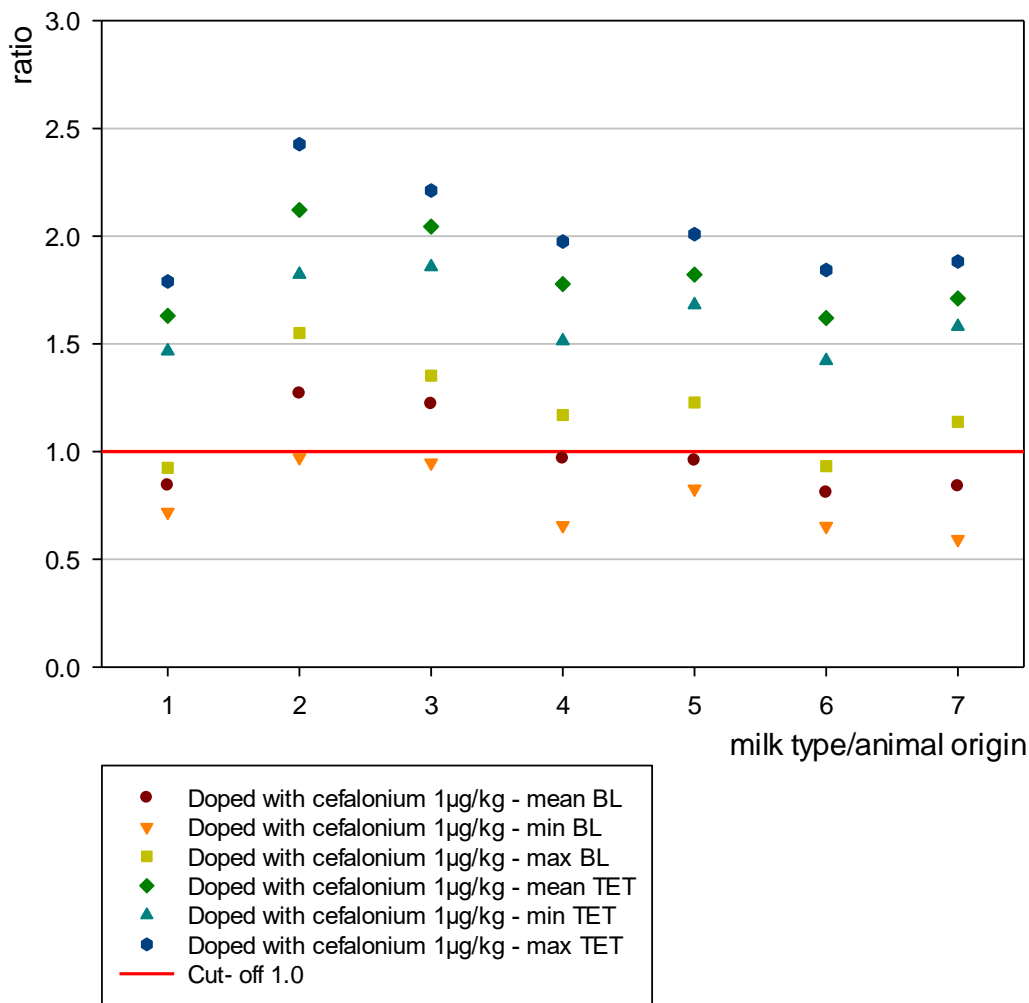

Fig. 9. Results for milk spiked with cefalonium at 1 µg/kg, 10 replicates. BL: β-lactam channel; TET: Tetracycline channel.

Table 14. AnticFast® Beta-lactams & Tetracyclines Combo Test Kit results for blank and spiked normal raw cows' milk and for blank and spiked milk of special composition (10 replicates).

	Ratio					
	Beta-lactam channel			Tetracycline channel		
	mean	min	max	mean	min	max
Blank milk						
normal raw cows' milk = reference	1.8827	1.7166	2.1104	1.6867	1.5789	1.8818
UHT milk*	2.5026	2.1936	2.9230	2.0522	1.8282	2.2808
sterilized milk*	2.3607	2.0768	2.5616	1.9719	1.7017	2.1978
milk powder*	2.1436	1.6979	2.7968	1.7780	1.4617	2.1298
thawed milk*	2.0561	1.7545	2.3628	1.9123	1.6990	2.1491
goats' (blank n=20)	1.6073	1.3404	2.0334	1.6268	1.4296	1.9921
ewes' (blank n=20)*	2.0142	1.2847	7.5528	1.9298	1.4603	5.9998
Milk with benzylpenicillin at 1 µg/kg + oxytetracycline at 3 µg/kg						
normal raw cows' milk = reference	0.6915	0.6140	0.7447	0.8499	0.7271	0.9018
UHT milk*	**1.0568**	0.9237	**1.2284**	0.7882	0.7158	0.9357
sterilized milk	**1.1460**	0.9731	**1.4714**	0.6431	0.4989	0.9974
milk powder*	0.7939	0.4832	0.9694	0.6971	0.4770	0.7976
thawed milk	0.6667	0.4246	0.7689	0.7853	0.6848	0.9063
goats' milk	0.6856	0.5742	0.8037	0.4939	0.3542	0.6009
ewes' milk	0.7484	0.6034	0.9192	0.7031	0.6168	0.7687
Milk with cefalonium at 1 µg/kg						
normal raw cows' milk = reference	0.8421	0.7185	0.9242	1.6303	1.4669	1.7905
UHT milk*	**1.2694**	0.9723	**1.5513**	2.1217	1.8226	2.4272
sterilized milk	**1.2199**	0.9475	**1.3530**	2.0450	1.8582	2.2121
milk powder*	0.9675	0.6572	**1.1692**	1.7778	1.5144	1.9753
thawed milk*	0.9590	0.8265	**1.2275**	1.8218	1.6815	2.0094
goats' milk	0.8097	0.6535	0.9319	1.6202	1.4228	1.8429
ewes' milk	0.8391	0.5930	**1.1376**	1.7114	1.5806	1.8823

Notes: *: decreased flow possible; min: minimum; max: maximum.

The AnticFast® Beta-lactams & Tetracyclines Combo Test Kit showed an interference of detection at the tested low concentrations of residues. However, it could be expected that the test kit could be used to analyse thawed milk and reconstituted milk powder, but no guarantee could be given for detection of ampicillin and amoxicillin at MRL in heat-treated milk such as UHT and sterilized milk.

For several samples of milk products or milk different from raw cows' milk, a diminished flow was observed. It is therefore always important to check that the milk has flown over the control line before interpreting the results.

The test can also be used to screen goats' and ewes' milk but in ewes' milk there is no guarantee that the same detection capabilities as determined in cows' milk could be obtained in ewes' milk.

6.5. Stability of reagents – daily control samples

Methods and material:
The following control samples were analyzed daily with the AnticFast® Beta-lactams & Tetracyclines Combo Test Kit to check the stability of the reagents and consistency of results:
- Blank milk (antibiotic-free raw milk)
- Raw milk spiked with 1 µg/kg of benzylpenicillin and oxytetracycline at 3 µg/kg
- Raw milk spiked with 1 µg/kg of cefalonium

Each day, also a negative and positive control (lot numbers and expiry dates are equal to those of the kit reagents (lot 1: 20200826G (expiration date 26/08/2021), lot 2: 20200812G (expiration date 12/08/2021)) as provided in the kit were analysed. These controls were dissolved in 2 ml of HPLC water. The positive control contained 3 µg/kg of benzylpenicillin and 25 µg/kg of oxytetracycline.

Results:
The results of the daily control samples and negative and positive control samples are presented in Figures 10 to 12. A summary of the results is provided in Table 15.

Table 15. AnticFast® Beta-lactams & Tetracyclines Combo Test Kit results (ratio values) for the daily standards and negative and positive control samples.

Standard	Ratio							
	Beta-lactam channel				Tetracycline channel			
	mean	min	max	s_r	mean	min	max	s_r
Blank milk								
	1.7277	1.2446	2.2385	0.20	1.6392	1.3228	2.0507	0.14
Milk spiked with benzylpenicillin at 1 µg/kg and oxytetracycline at 3 µg/kg								
	0.3582	0.0795	0.6129	0.13	0.7007	0.1095	0.8515	0.16
Milk spiked with cefalonium at 1 µg/kg								
	0.7010	0.6172	0.8816	0.10	1.6502	1.4900	1.8582	0.11
Controls included in the test kit								
Negative	1.2414	**0.9243***	1.3115	0.09	1.2667	1.0505	1.3073	0.06
Positive	0.0869	0.0705	0.1102	0.01	0.1181	0.1050	0.1367	0.01

Notes: *: negative due to an interruption of the β-lactam test line; S_r: standard deviation; min: lowest ratio; max: highest ratio.

Discussion:
Very stable ratio values were obtained for daily control samples with the AnticFast® Beta-lactams & Tetracyclines Combo Test Kit reagents over the test period. Correct values were obtained for the different daily standards: all blank milk standards gave a negative result on all channels. The milk samples spiked with a concentration of 1 µg/kg of benzylpenicillin and 3 µg/kg of tetracycline and the milk samples spiked with a concentration of 1 µg/kg of cefalonium always resulted in positive results.
For the last 4 results for blank milk and last 2 results for both spiked milk samples, the improved reagents (lot 20210921G (expiration date 21/09/2022) and lot 20211008G (expiration date

08/10/22)) were used. For the doped samples with benzylpenicillin at 1 µg/kg and oxytetracycline at 3 µg/kg, much lower (more positive) results were obtained on both channels. The negative and positive controls inserted in the kit always gave correct results. One false positive result was noted but visually could be observed that the test line was interrupted in the middle.
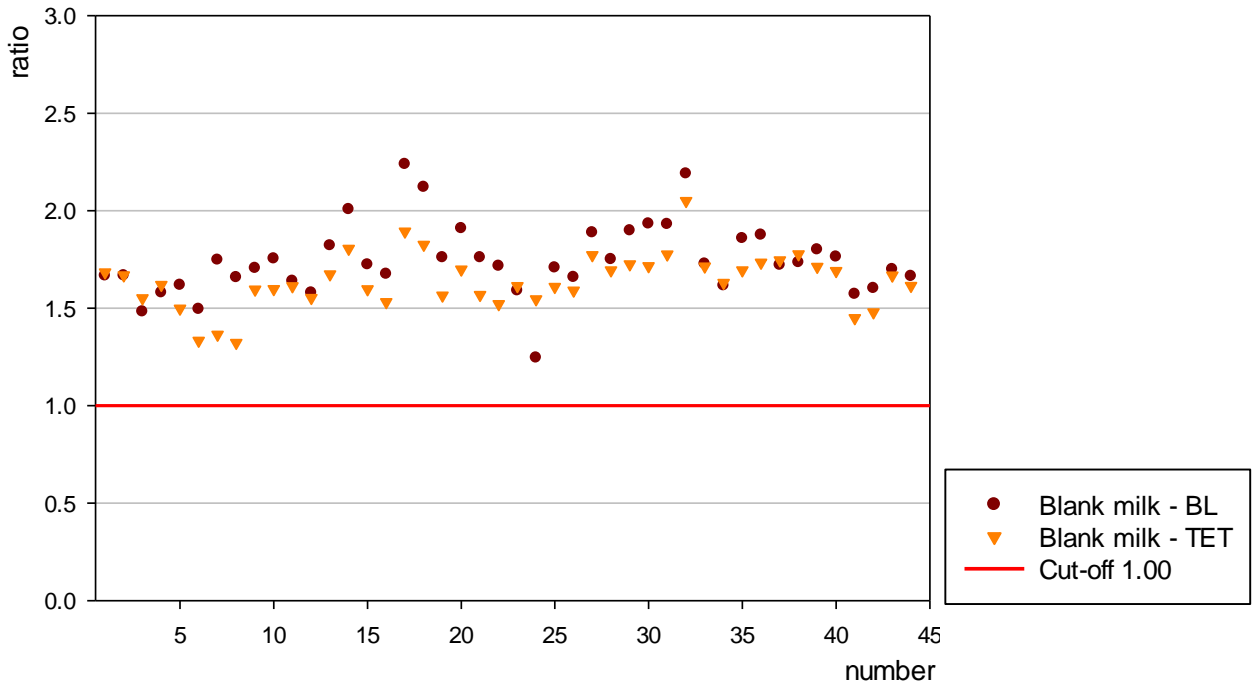

Fig. 10. AnticFast® Beta-lactams & Tetracyclines Combo Test Kit results (ratio) for the blank control samples.

Fig. 11. AnticFast® Beta-lactams & Tetracyclines Combo Test Kit results (ratio) for the spiked control samples with benzylpenicillin at 1 µg/kg and oxytetracycline at 3 µg/kg and spiked with cefalonium at 1 µg/kg.
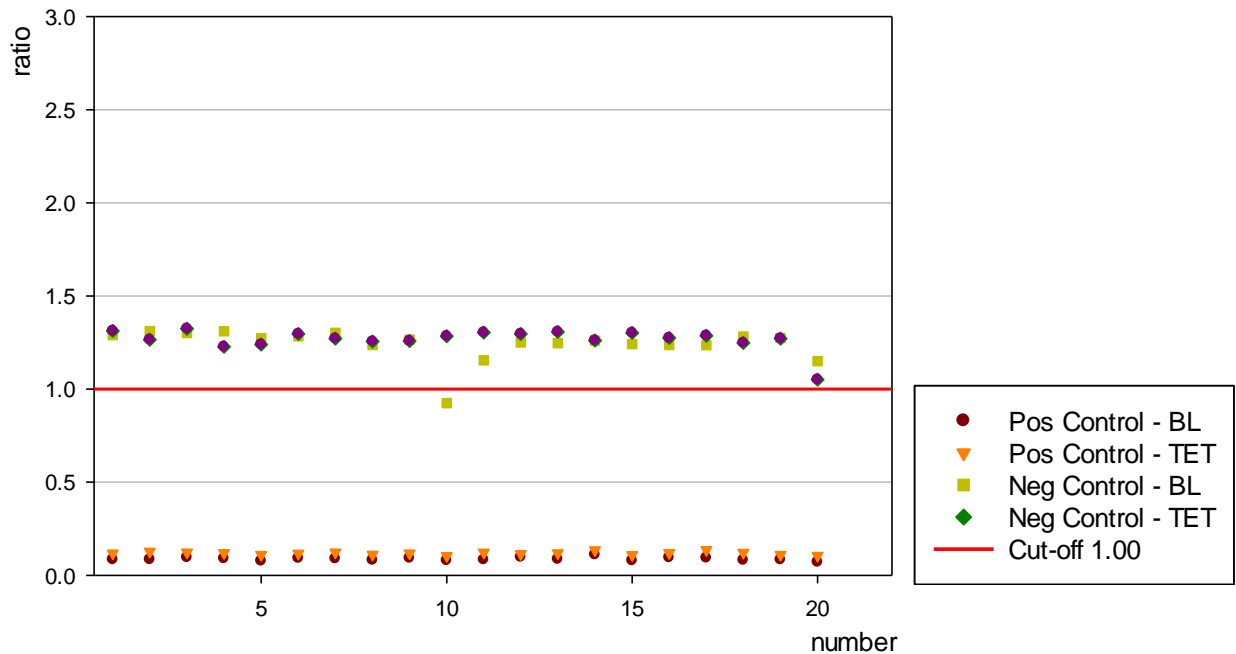
Fig. 12. AnticFast® Beta-lactams & Tetracyclines Combo Test Kit results (ratio) for the negative and positive controls inserted in the kit.

7. Reliability of the instrumentation

The software of the reader could be improved: presently the ID code doesn't change so looking up previous results is difficult.
The reader doesn't indicate invalid results when the control line is not well formed.

8. Interlaboratory testing - 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 trial of spring 2021, AnticFast® Beta-lactams & Tetracyclines Combo Test Kit as integrated as rapid test.

Results:
The results obtained in the interlaboratory study of April 22, 2021 are shown in Table 16.

Table 16. AnticFast® Beta-lactams & Tetracyclines Combo Test Kit results in the interlaboratory study of April 22, 2021.

LAB	Visual reading AnticFast® Beta-lactams & Tetracyclines Combo Test Kit								Instrumental reading (ratio + result) AnticFast® Beta-lactams & Tetracyclines Combo Test Kit								Lot number Expiry date Type of reader
	A	B	C	D	E	F	G	H	A	B	C	D	E	F	G	H	
TETRACYCLINES																	20200812G 12/08/2021 BMZ6000 Portable Strip Reader
ILVO	-	-	-	-	+	-	-	-	1.8429 NEG	1.8183 NEG	1.7690 NEG	1.7716 NEG	0.1653 POS	1.7066 NEG	1.8050 NEG	1.4643 NEG	
BETA-LACTAM																	
ILVO	-	+	+	+	-	-	-	+	1.9315 NEG	0.6603 POS	0.1947 POS	0.2113 POS	1.3797 NEG	1.6066 NEG	1.9949 NEG	0.7873 POS	

Notes: The cut-off value of the BMZ6000 Portable Strip-Reader is 1.0000. Milk samples generating a ratio below this cut-off value (< 1.0000) are considered positive.

Discussion:

Good results were obtained with AnticFast® Beta-lactams & Tetracyclines Combo Test Kit.

Except for sample F, spiked with 100 ppb cefalexin, all other milk samples fortified with ß-lactam antibiotics (samples B, C, D & H) were screened positive with AnticFast® Beta-lactams & Tetracyclines Combo Test Kit. This is in line with the detection capability reported by Meizheng Group for cefalexin (> MRL) and the ILVO validation results. The milk sample spiked with 100 ppb chlortetracycline (sample E) was screened positive on the tetracycline test line of AnticFast® Beta-lactams & Tetracyclines Combo Test Kit.
Hence, in this ring test cefquinome, cloxacillin, benzylpenicillin, chlortetracycline and ampicillin are detected at MRL with AnticFast® Beta-lactams & Tetracyclines Combo Test Kit.

Negative results were obtained for the blank milk (sample G) on both channels and also for the milk samples doped with antibiotics that are supposed to give a negative result (on the respective test lines). So, there were no false positive results with AnticFast® Beta-lactams & Tetracyclines Combo Test Kit.

9. Final conclusion

Results of this validation show that the AnticFast® Beta-lactams & Tetracyclines Combo Test Kit is an easy, reliable, robust and highly specific test for screening of raw cows' milk for residues of β-lactam antibiotics (penicillins and cefalosporins) and tetracyclines. With the improved reagents (lot 20210921G (expiration date 21/09/2022) and lot 20211008G (expiration date 08/10/22)), all β-lactams can be detected at least in 95% of the replicates at their respective MRL except for desfuorylceftiofur and cefalexin. All tetracyclines (parent drugs and 4-epimers) can be detected at least in 95% of the replicates at their respective MRL. Doxycycline, not for use in animals from which milk is produced for human consumption, can be detected at least in 95% of the replicates from 2 µg/kg on.
In general the test showed to be robust against small changes in the test protocol and milk composition/quality. Just the detection of β-lactams could be slightly hampered by a high protein content of the milk.

The test is suitable to test other types of milk, such as thawed milk and reconstituted milk powder. AnticFast® Beta-lactams & Tetracyclines Combo Test Kit could also be used to screen goats' milk. When testing heat-treated milk (UHT, sterilized) or ewes' milk, higher detection capabilities are possible compared to testing of raw cows' milk.

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