Validation report AnticFast® Beta-lactams & Tetracyclines & Cefalexin Rapid Test Kit

(Order n°: JC0641)

(Meizheng Bio-Tech, China)

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1. Introduction

AnticFast® Beta-lactams & Tetracyclines & Cefalexin Rapid Test Kit (Meizheng Bio-Tech, China) is a qualitative two-step (3 min + 5 min) rapid lateral flow assay to detect β-lactam (penicillins and cefalosporins), including cefalexin 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) according to Commission Decision 2002/657/EC and to the 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 included in the interlaboratory study organised by ILVO in spring 2021.

Update of the report: determination of the detection capacity for ceftiofur with improved reagents (lot 3: 20210909G (expiration date 09/09/2022) and lot 4: 20211009G (expiration date 09/10/22). Also extra blank farm and 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: 20201201G (expiration date 01/12/2021) and lot 2: 20201215G (expiration date 15/12/2021). The results of the age of reagents testing of lot 1 were also included.

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). In this validation study, raw milk temperature was standard 1-4°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
Step 1: Turn on the incubator and wait until it is stable at 40°C. Place the empty microwells into the incubator.
Step 2: 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.
Step 3: Incubate the sample for 3 minutes at 40°C, then insert the test strip into the microwell.
Step 4: Let the test strip develop color for 5 minutes at 40°C.
Step 5: 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 to T3) 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 & Cefalexin Rapid Test Kit test strip

The configuration of the AnticFast® Beta-lactams & Tetracyclines & Cefalexin Rapid Test Kit is shown in Figure 1.
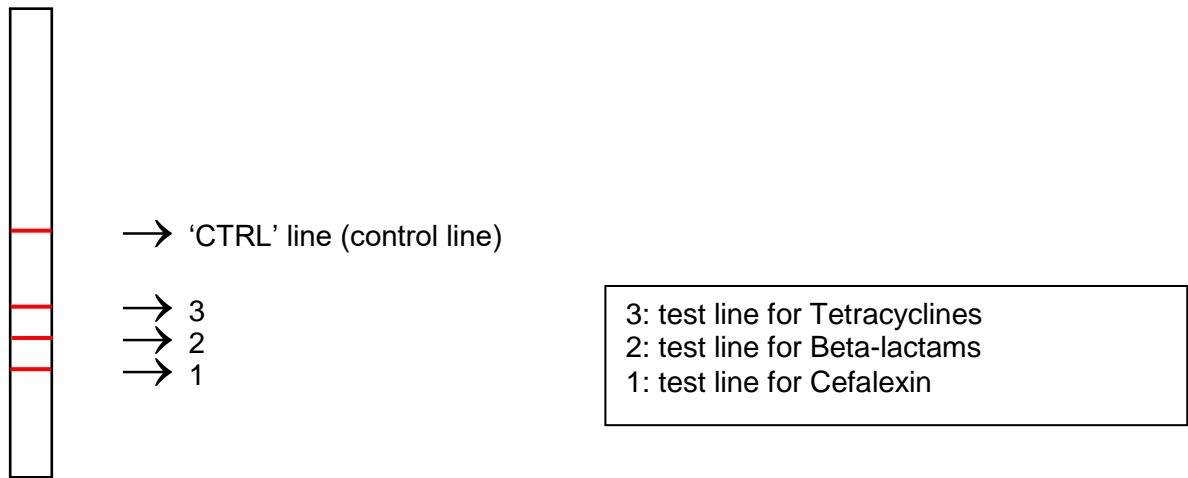
→ 'CTRL' line (control line)

→ 3
→ 2
→ 1

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

Fig. 1. Configuration of an AnticFast® Beta-lactams & Tetracyclines & Cefalexin Rapid 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. HMG-GS Microwell Incubator and BMZ6000 Portable Strip Reader for instrumental reading of the color formation on the AnticFast® Beta-lactams & Tetracyclines & Cefalexin Rapid Test Kit test strips.

2.3 Visual interpretation of the test

The kit manufacturer claims that visual reading of the AnticFast® Beta-lactams & Tetracyclines & Cefalexin Rapid Test Kit test strips 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.
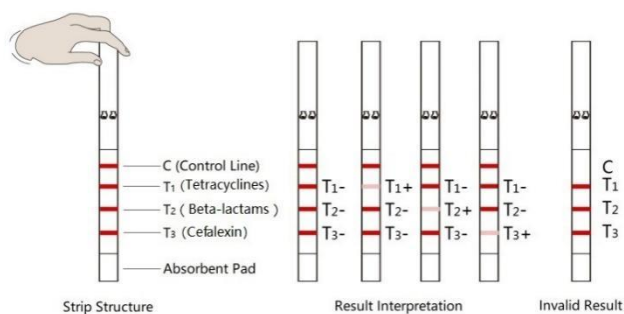
Fig. 3. Visual interpretation of the color formation on the AnticFast® Beta-lactams & Tetracyclines & Cefalexin Rapid Test Kit test strips(*Anon.*; 2020).

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 & Cefalexin Rapid 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 4 µg/kg - twice
- blank raw milk spiked with cefalexin at 4 µg/kg – twice

Detection capability tests were performed with 4 different lots of reagents: lot 1 20201201G (expiration date 01/12/2021) and lot 2 20201215G (expiration date 15/12/2021), and for the detection capability of ceftiofur, two extra (more sensitive) lots were used: lot 3 20210909G (expiration date 09/09/2022) and lot 4 20211009G (expiration date 09/10/22). 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.

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

Table 3. Standard material used in this validations study.

Compound	Origin	Product number	Lot number
Amoxicilline trihydrate	Sigma Aldrich	31586	BCCB1309
Ampicillin trihydrate	Sigma Aldrich	31591	BCBS3642V
Cefacetrile	LGC Standards	C231500	2-MAX-147-3
Cefalexine VETRANAL	Sigma Aldrich	33989	BCBW7031
Cefalonium hydrate	Sigma Aldrich	32904	BCBV1595
Cefapirin sodium	Sigma Aldrich	43989	BCCC5208
Cefazoline European Pharmacopoeia Reference Standard	Sigma Aldrich	C0682800	5.0
Cefoperazone dihydrate	Sigma Aldrich	32426	BCBX0019
Cefquinome sulfate	LGC Standards	C16998175	G1005777
Ceftiofur	Dr. Ehrenstorfer	DRE-C11065000	G1104213
Chloramphenicol VETRANAL	Sigma Aldrich	31667	BCBR6685V
Chlortetracycline hydrochloride	Sigma Aldrich	46133	BCBT9837
Clavulaanzuur (Potassium clavulante)	Sigma Aldrich	33454	STBJ0056
Cloxacilline sodium salt monohydrate VETRANAL	Sigma Aldrich	46140	BCBW1061
Colistin sulfate	Sigma Aldrich	C4461	049M4836V
Dapsone	Sigma Aldrich	46158	BCBX0187
Desacetylcephapirin sodium salt	LGC standards	682120	799058
Desfuroylceftiofur	TRC	D289980	5-WBZ-57-5
Dicloxacilline	Sigma Aldrich	46182	BCBX4662
Doxycycline Hyclate VETRANAL	Sigma Aldrich	33429	BCBS7684V
Doxycycline Hyclate	LGC Standards	C13084280	1116543
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	BCBX0881
4- epimer chlortetracycline hydrochloride	Acros Organics	268231000	A0406408
4- epimer oxytetracycline	Acros Organics	257711000	A0395560
4- epimer tetracycline hydrochloride	Acros Organics	233121000	A0397675

Results:

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

Table 4. Detection capability (in µg/kg) of AnticFast® Beta-lactams & Tetracyclines & Cefalexin Rapid Test Kit (Meizheng Bio-Tech, 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	3	
amoxicillin	4	3	
oxacillin	30	3	
cloxacillin	30	3	
dicloxacillin	30	2	
nafcillin	30	16	
Cefalosporins			
ceftiofur	100[a]		35
desfuroylceftiofur	100[a]	-- (>MRL)*	
cefquinome	20	9	
cefazolin	50	45	
cephapirin	60[b]	9	
desacetylcephapirin	60[b]	35	
cefacetrile	125	30	
cefoperazone	50	2	
cefalexin	100	3**	
cefalonium	20	1	
Tetracyclines			
tetracycline	100[c]	3	
4-epimer of tetracycline	100[c]	6	
oxytetracycline	100[c]	3	
4-epimer of oxytetracycline	100[c]	6	
chlortetracycline	100[c]	10	
4-epimer of chlortetracycline	100[c]	20	
doxycycline	100[d]	2	

Notes: lot 1 20201201G (expiration date 01/12/2021), lot 2 20201215G (expiration date 15/12/2021), lot 3 20210909G (expiration date 09/09/2022) and lot 4 20211009G (expiration date 09/10/22).

*: detection capability >MRL, exact detection capability not tested; **: cefalexin test line. 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 & Cefalexin Rapid 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 of which the 95% detection capability was not determined as it could not be detected at MRL.

It's worth noting that ceftiofur could not be detected at MRL with the first two lots of reagents. Based on this information the kit manufacturer decided to adjust the reagents (lots 3 and 4). With these new improved reagents a CCβ of 35 µg/kg was determined for ceftiofur. The impact of the improvement of the reagents on the CCβ for the other β-lactams was not determined.

All tetracyclines and their 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.

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 & Cefalexin Rapid 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, cefalexin and tetracycline 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), cefalexin and 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.

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

Family	Compound	MR(P)L (µg/kg)	Conc. spiked in milk (µg/kg)	Tet channel		Beta channel		Cefa channel	
						Ratio	Result	Ratio	Result
Penicillins	Benzylpenicillin	4	400	1.9102	-	0.1180	+	1.9479	-
Cefalosporins	Cefalexin	100	10,000	1.8604	-	**0.2253**	+	0.1699	+
			5,000	2.1358	-	2.3876	-	0.3215	+
	Cefalonium	20	2,000	1.9016	-	0.0984	+	1.8828	-
Tetracyclines	Oxytetracycline	100[a]	10,000	0.0728	+	2.1603	-	2.0754	-
Sulfonamides	Sulfadiazine	100[b]	10,000	2.0585	-	2.6679	-	2.2737	-
Aminoglycosides	Neomycin B	1,500	150,000	2.1075	-	2.4089	-	2.3523	-
Macrolides	Erythromycin	40	4,000	2.0928	-	2.3734	-	2.1513	-
Quinolones	Enrofloxacin	100[c]	10,000	2.0973	-	2.4742	-	2.1947	-
Amphenicols	Chloramphenicol	0.3[d]	30	2.0580	-	2.3984	-	2.1742	-
Polymyxins	Colistin	50	5,000	2.0158	-	2.3342	-	2.1544	-
Lincosamides	Lincomycin	150	15,000	2.0112	-	2.2726	-	2.0530	-
β-lactamase inhibitors	Clavulanic acid	200	20,000	2.1084	-	**0.1615**	**+**	2.2045	-
Diamino pyrimidine derivatives	Trimethoprim	50	5,000	2.0061	-	2.2970	-	2.1251	-
Others	Dapsone	5[e]	500	2.2258	-	2.4802	-	2.3999	-

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; Tet: tetracycline; Beta: β-lactam;. Cefa: cefalexin.
[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).

Discussion:

On the β-lactam channel, as expected, cefalexin (beta-lactam) gave also positive results in high concentrations (100×MRL). But cefalexin at 5,000 µg/kg (50×MRL) tested negative on the β-lactam channel.

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 1,000 µg/kg on.

The specificity of the cefalexin channel was not tested. So the possibility to get a positive result on the cefalexin channel caused by high concentrations of a β-lactam compound different from cefalexin could not be excluded.

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

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

Methods and materials:
302 blank farm and 300 tanker load milk samples were tested with AnticFast® Beta-lactams & Tetracyclines & Cefalexin Rapid Test Kit lot 1 and 2 and other microbiological and receptor screening tests.
With the improved reagents of lot 3 and 4, 49 additional blank farm and 49 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 302 farm milk samples, 300 tested negative for β-lactams and tetracyclines on AnticFast® Beta-lactams & Tetracyclines & Cefalexin Rapid Test Kit. Two farm milk samples tested positive on the β-lactam channel, but were proven to be real positives by use of other screening tests. Of the 300 tanker load milk samples all tested negative for β-lactams and tetracyclines. Giving these results, it is concluded that in total no false positive results were obtained upon 600 samples on both test channels. The results are summarized in Table 6a.

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

Lot 1 and 2	Farm milk (n=300)			Tanker milk (n=300)		
	Ratio			Ratio		
	Tet	Beta	Cefa	Tet	Beta	Cefa
Mean	2.1334	2.5416	2.2158	2.2379	2.7312	2.2169
Min	1.1645	1.9415	1.6930	1.7644	2.2254	1.6412
Max	2.4750	3.1284	2.6808	2.5675	3.0849	2.7453
s_r	0.12	0.16	0.15	0.11	0.15	0.15
CV%	5.73	6.18	6.58	5.03	5.59	6.77

Notes: mean: mean ratio; min: lowest ratio; max: highest ratio; Tet: tetracycline; Beta: β-lactam and Cefa: cefalexin. s_r ; Standard Deviation; CV(%): Relative Standard Deviation.

For the improved reagents (lot 3 and 4): Of the 49 farm milk and 49 tanker load milk samples, all tested negative for β-lactams, including cefalexin and tetracyclines on AnticFast® Beta-lactams & Tetracyclines & Cefalexin Rapid Test Kit. Giving these results, it is concluded that in total no false positive results were obtained upon 98 samples on all test channels. The results are summarized in Table 6b.

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

Lot 3 and 4	Farm milk (n=49)			Tanker milk (n=49)		
	Ratio			Ratio		
	Tet	Beta	Cefa	Tet	Beta	Cefa
Mean	1.7155	1.4946	1.9380	1.7433	1.5210	1.9452
Min	1.6183	1.3238	1.7859	1.5497	1.1950	1.7844
Max	1.8571	1.6450	2.0947	1.8423	1.6807	2.0563
s_r	0.06	0.08	0.08	0.06	0.09	0.07
CV%	3.61	5.53	4.20	3.35	6.13	3.64

Notes: mean: mean ratio; min: lowest ratio; max: highest ratio; Tet: tetracycline; Beta: β-lactam and Cefa: cefalexin. s_r ; Standard Deviation; CV(%): Relative Standard Deviation.

5. Reader and test repeatability
5.1 Repeatability of the reader

Methods and Materials:
Samples of 10 blank,10 low positive samples and 10 high positive samples for each channel were measured twice. For the spiked samples, any compound found positive could be used for the testing of the reader repeatability.

Results:

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

Table 7. Repeatability of the reader

Reader repeatability	Tetracyclines			Beta-lactam			Cefalexin		
	Mean	s_r	CV%	Mean	s_r	CV%	Mean	s_r	CV%
Blank milk	2.1453	0.01	0.47	2.5155	0.01	0.53	2.2497	0.01	0.63
Low positive milk	0.8663	0.00	0.12	0.8923	0.00	0.31	0.8588	0.00	0.32
High positive milk	0.2966	0.00	0.44	0.2943	0.00	1.01	0.4856	0.01	2.36

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

Discussion:

The repeatability of the reader was very good; very low relative standard deviations were obtained (highest value 2.36%).

5.2 Repeatability of the test

Methods and Materials:

Twin samples of 10 blank,10 low positive samples and 10 high positive samples for each channel were analysed. For the spiked samples, any compound found positive could be used for the testing of channel.

Results:

The results of the repeatability of the AnticFast® Beta-lactams & Tetracyclines & Cefalexin Rapid 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	Tetracyclines			Beta-lactam			Cefalexin		
	Mean	s_r	CV%	Mean	s_r	CV%	Mean	s_r	CV%
Blank milk	2.1430	0.03	1.34	2.5136	0.07	2.62	2.2695	0.08	3.58
Low positive milk	0.8690	0.02	1.89	0.8672	0.03	3.74	0.8434	0.04	4.79
High positive milk	0.3595	0.04	10.33	0.3058	0.01	2.91	0.4448	0.03	5.88

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

Discussion:
The repeatability of the test was also good, low relative standard deviation values were obtained. The highest variance value is 10.33%.

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 incubation steps and 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 3+5 minutes.

Methods and Materials:
Blank and spiked milk samples containing benzylpenicillin at 1 µg/kg and oxytetracycline at 4 µg/kg or spiked milk containing cefalexin at 4 µg/kg were analysed (3 replicates) with a test protocol with incubation timings different from the test protocol (reference = 3'+5').

Results:
The results of the influence of the length of the incubation steps on the AnticFast® Beta-lactams & Tetracyclines & Cefalexin Rapid 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 benzylpenicillin at 1 µg/kg and oxytetracycline at 4 µg/kg did show slightly lower mean ratios (less variation between ratios) on the β-lactam channel when deviating the length of the incubation from the standard 3' + 5', giving slightly more positive results.

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

Length of incubation steps	Blank milk			Milk spiked with benzylpenicillin at 1 μg/kg and oxytetracycline at 4 μg/kg			Milk spiked with cefalexin at 4 μg/kg		
	Tet	Beta	Cefa	Tet	Beta	Cefa	Tet	Beta	Cefa
2 minutes 45 seconds + 4 minutes 30 seconds									
mean	2.2239	2.6710	2.2991	0.7250	0.3715	2.0986	2.2080	2.6765	0.7549
min	2.1439	2.5487	2.2149	0.6875	0.3482	2.0615	2.1907	2.6476	0.7477
max	2.2844	2.7718	2.3900	0.7578	0.4018	2.1719	2.2205	2.6976	0.7659
2 minutes 45 seconds + 5 minutes									
mean	2.2252	2.6943	2.3042	0.7149	0.3339	1.9875	2.1734	2.6152	0.7679
min	2.1908	2.6128	2.2762	0.6926	0.2730	1.9587	2.1346	2.5799	0.7624
max	2.2480	2.7627	2.3195	0.7430	0.3698	2.0247	2.2067	2.6371	0.7777
2 minutes 45 seconds + 5 minutes 30 seconds									
mean	2.1631	2.5570	2.1658	0.7096	0.3678	2.0453	2.1422	2.5636	0.7160
min	2.0963	2.4770	2.0323	0.6642	0.3014	1.9253	2.0834	2.4971	0.6821
max	2.2928	2.6883	2.2941	0.7602	0.7097	2.1151	2.2313	2.6465	0.7381
3 minutes + 4 minutes 30 seconds									
mean	2.2379	2.6854	2.2657	0.7168	0.3510	2.1159	2.2141	2.6951	0.7373
min	2.1884	2.5900	2.1722	0.6984	0.3023	2.0902	2.1675	2.6282	0.7321
max	2.2699	2.7506	2.3314	0.7457	0.3815	2.1444	2.2886	2.7915	0.7431
3 minute + 5 minutes (REF)									
mean	2.2204	2.7012	2.3099	0.7801	0.4350	1.9585	2.2226	2.7102	0.7567
min	2.2006	2.6442	2.2508	0.6831	0.2825	1.9351	2.1859	2.6694	0.6951
max	2.2352	2.7959	2.3684	0.9557	0.6855	1.9766	2.2843	2.7512	0.7998
3 minutes + 5 minutes 30 seconds									
mean	2.0796	2.4336	2.0975	0.7238	0.3061	1.9510	2.1236	2.4994	0.6824
min	1.9595	2.2655	1.9086	0.6786	0.2679	1.9329	2.0387	2.3928	0.6644
max	2.2319	2.6765	2.3157	0.7597	0.3285	1.9729	2.1986	2.5687	0.6957
3 minute 15 seconds + 4 minutes 30 seconds									
mean	2.3032	2.8350	2.4320	0.7452	0.3182	2.0927	2.1828	2.6950	0.7400
min	2.2096	2.6479	2.2693	0.7168	0.2981	2.0274	2.1734	2.6420	0.6981
max	2.4174	2.9799	2.5788	0.7824	0.3304	2.1546	2.1953	2.7453	0.7707
3 minute 15 seconds + 5 minutes									
mean	2.0953	2.4610	2.0867	0.7165	0.3105	2.1201	2.1556	2.5623	0.7100
min	2.0661	2.4322	2.0117	0.6789	0.2588	2.1055	2.0578	2.4238	0.6691
max	2.1360	2.5075	2.1633	0.7604	0.3397	2.1313	2.2637	2.7092	0.7780
3 minute 15 seconds + 5 minutes 30 seconds									
mean	2.0369	2.3676	2.0205	0.7260	0.2700	1.9452	2.0557	2.4434	0.7086
min	1.9840	2.3004	1.9621	0.7066	0.2171	1.8871	2.0432	2.4011	0.6705
max	2.1195	2.4675	2.1011	0.7629	0.3203	1.9982	2.0619	2.5019	0.7334

Notes: REF: reference; mean: mean ratio; min: minimum ratio; max: maximum ratio; Tet: Tetracycline; Beta: β-lactam; Cefa: Cefalexin.

6.1.2. Delay of reading

Methods and Materials:

Blank and spiked milk samples containing benzylpenicillin at 1 μg/kg and oxytetracycline at 4 μg/kg or spiked milk containing cefalexin at 4 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 & Cefalexin Rapid Test Kit strips directly after incubation or with a delay of 5 or 10 minutes respectively.

Delay of reading	Ratio								
	No delay (REF)			5 min			10 min		
	Tet	Beta	Cefa	Tet	Beta	Cefa	Tet	Beta	Cefa
Blank milk									
mean	2.2204	2.7012	2.3099	2.1055	2.6021	2.1712	1.9116	2.3589	1.9298
min	2.2006	2.6442	2.2508	2.0718	2.5646	2.1354	1.8188	2.2026	1.8078
max	2.2352	2.7959	2.3684	2.1382	2.6719	2.2008	1.9735	2.4637	1.9940
Milk spiked with benzylpenicillin at 1 μg/kg and oxytetracycline at 4 μg/kg									
mean	0.7801	0.4350	1.9585	0.7775	0.3907	1.8090	0.7350	0.3749	1.6913
min	0.6831	0.2825	1.9351	0.6458	0.2385	1.7022	0.6533	0.2736	1.6656
max	0.9557	0.6855	1.9766	0.9710	0.6356	1.8868	0.8943	0.5718	1.7184
Milk spiked with cefalexin at 4 μg/kg									
mean	2.2226	2.7102	0.7567	2.1323	2.5909	0.7402	1.9889	2.4384	0.6293
min	2.1859	2.6694	0.6951	2.0447	2.5105	0.6622	1.9804	2.4137	0.5938
max	2.2843	2.7512	0.7998	2.2249	2.6795	0.8089	1.9959	2.4627	0.6787

Notes: REF: reference; mean: mean ratio; min: minimum ratio; max: maximum ratio; Tet: Tetracycline; Beta: β-lactam; Cefa: Cefalexin.

Discussion:
Delay in reading the devices did not significantly impact the interpretation of test results: all negative results remained negative and all 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 4 μg/kg or spiked milk containing cefalexin at 4 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). On the beta-lactam and tetracycline channel, also a decreased milk volume gave slightly more positive results.

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

Milk volume	Ratio								
	180 μl			200 μl (REF)			220 μl		
	Tet	Beta	Cefa	Tet	Beta	Cefa	Tet	Beta	Cefa
Blank milk									
mean	2.1873	2.5869	2.1976	2.2204	2.7012	2.3099	2.2337	2.7056	2.2613
min	2.1243	2.5578	2.1650	2.2006	2.6442	2.2508	2.1616	2.5718	2.0490
max	2.2260	2.6222	2.2328	2.2352	2.7959	2.3684	2.3416	2.8506	2.4307
Milk with benzylpenicillin at 1 μg/kg and oxytetracycline at 4 μg/kg									
mean	0.7436	0.3476	1.9444	0.7801	0.4350	1.9585	0.7252	0.2832	2.0899
min	0.6767	0.3017	1.8861	0.6831	0.2825	1.9351	0.7016	0.2634	2.0327
max	0.8031	0.3857	1.9900	0.9557	0.6855	1.9766	0.7513	0.3054	2.1756
Milk with cefalexin at 4 μg/kg									
mean	2.1930	2.6123	0.7612	2.2226	2.7102	0.7567	2.1691	2.6664	0.7471
min	2.1484	2.5752	0.7166	2.1859	2.6694	0.6951	2.1087	2.5712	0.7192
max	2.2366	2.6715	0.8046	2.2843	2.7512	0.7998	2.2381	2.8049	0.7882

Notes: REF: reference; mean: mean ratio; min: minimum ratio; max: maximum ratio; Tet: Tetracycline; Beta: β-lactam; Cefa: Cefalexin.

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 and of 1-4°C (= reference) in order to check if the milk temperature is influencing the AnticFast® Beta-lactams & Tetracyclines & Cefalexin Rapid Test Kit result. Besides blank milk also spiked milk samples containing benzylpenicillin at 1 µg/kg and oxytetracycline at 4 µg/kg or spiked milk containing cefalexin at 4 µg/kg were used.

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

Discussion:
The milk temperature (20°C) did not significantly impact the AnticFast® Beta-lactams & Tetracyclines & Cefalexin Rapid 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 20°C, slightly lower ratios were obtained and a smaller range of variation for spiked milk.

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

Milk tempe-rature	Ratio					
	1-4°C (REF)			20°C		
	Tet	Beta	Cefa	Tet	Beta	Cefa
Blank milk						
mean	2.0326	2.3724	2.0720	2.1545	2.5325	2.2851
min	1.9567	2.2850	2.0054	2.0176	2.3585	2.1513
max	2.0991	2.4293	2.1268	2.2538	2.6285	2.3704
Milk with benzylpenicillin at 1 µg/kg and oxytetracycline at 4 µg/kg						
mean	0.7077	0.4953	1.9554	0.6599	0.3760	1.9374
min	0.6792	0.4771	1.8545	0.6379	0.3575	1.8302
max	0.7563	0.5070	2.0492	0.6773	0.3985	2.0207
Milk with cefalexin at 4 µg/kg						
mean	2.0907	2.4867	0.7561	2.1141	2.5181	0.7071
min	1.9970	2.3667	0.7092	1.9876	2.3396	0.6596
max	2.2213	2.6439	0.7835	2.1876	2.6402	0.7324

Notes: REF: reference; mean: mean ratio; min: minimum ratio; max: maximum ratio. Tet: tetracycline, Beta: β-lactam; Cefa: Cefalexin.

6.3. Milk composition and milk type influences

Methods and Materials:

Fat content
Normal milk samples and milk samples with a low (<0.91 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 & Cefalexin Rapid Test Kit result. The milk samples tested were natural milk samples with a low or high fat content with selected at the milk control station based on infrared spectroscopic results on a MilcoScan 4000 or the composition checked internally at ILVO with a Lactoscope FT-A.

Protein content
Normal milk samples and milk samples with a low (<3.04 g per 100 ml) or a high (>4.00 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 & Cefalexin Rapid 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 or the composition internally checked at ILVO with a Lactoscope FT-A.

Results:
With respect to the impact of the milk composition (fat content and protein content), the mean, the highest and lowest reader value are given in Figures 4 to 6 and Table 13.

The legend for the different situations in Figure 4 to 6.
 1 = Reference: normal raw milk;
 2 = Low fat content (<0.91 g/100 ml);
 3 = High fat content (>6.01 g/100 ml);
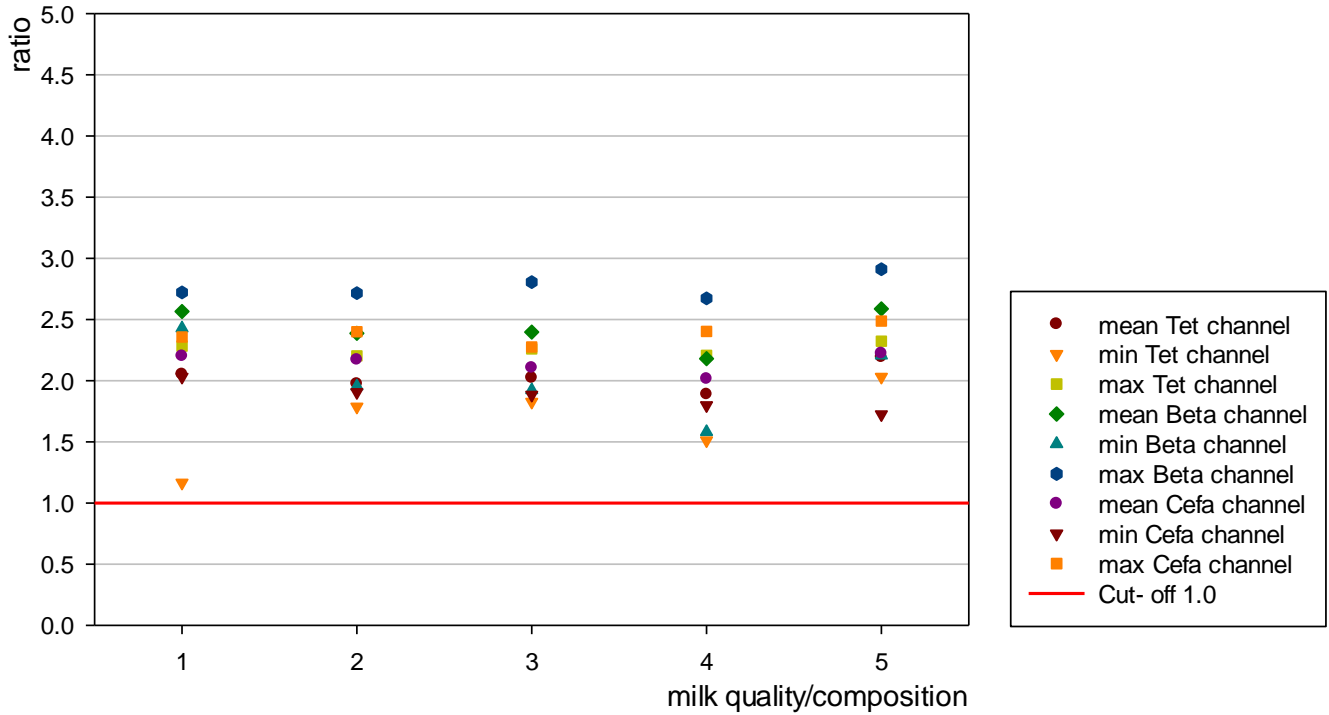 4 = Low protein (<3.04 g/100 ml);
 5 = High protein (>4.00 g/100 ml);

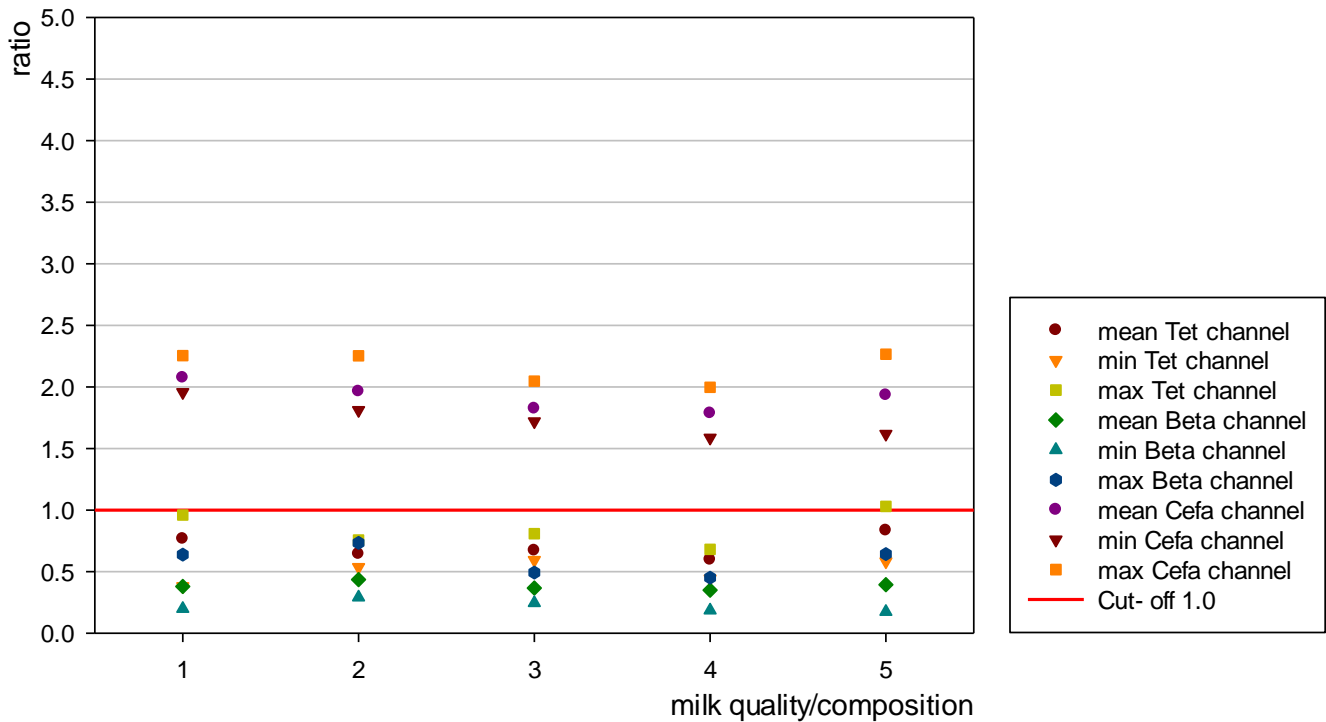Fig. 4. Results for blank milk, 10 replicates. Tet: Tetracycline; Beta: β-lactam; Cefa: Cefalexin.

Fig. 5. Results for milk spiked with benzylpenicillin at 1 µg/kg and oxytetracycline at 4 µg/kg, 10 replicates. Tet: Tetracycline; Beta: β-lactam; Cefa: Cefalexin.
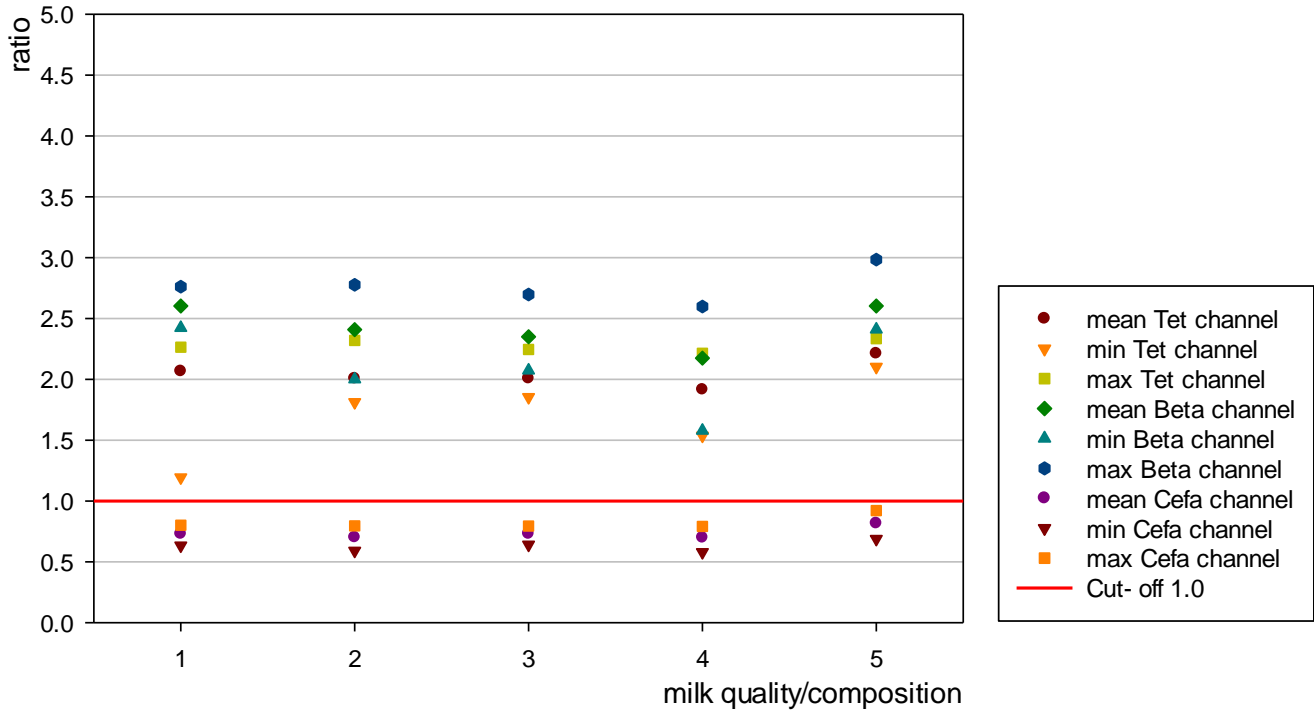
Fig. 6. Results for milk spiked with cefalexin at 4 µg/kg, 10 replicates. Tet: Tetracycline; Beta: β-lactam; Cefa: Cefalexin.

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

| | Ratio | | | | | | | | |
| | Tet | | | Beta | | | Cefa | | |
	mean	min	max	mean	min	max	mean	min	max
Blank raw cows' milk									
Normal milk = reference	2.0507	1.1645	2.2831	2.5663	2.4298	2.7215	2.2003	2.0307	2.3583
Low fat < 0.91g/100ml	1.9721	1.7860	2.2036	2.3873	1.9538	2.7165	2.1712	1.9057	2.4008
High fat > 6.01g/100ml	2.0220	1.8260	2.2606	2.3975	1.9217	2.8065	2.1037	1.8829	2.2764
Low protein < 3.04g/100ml	1.8864	1.5101	2.2078	2.1808	1.5801	2.6732	2.0138	1.7976	2.4039
High protein > 4.00g/100ml	2.1942	2.0304	2.3237	2.5877	2.2072	2.9122	2.2233	1.7229	2.4872
Milk with benzylpenicillin at 1 µg/kg and oxytetracycline at 4 µg/kg									
Normal milk = reference	0.7655	0.3812	0.9590	0.3787	0.1974	0.6381	2.0738	1.9558	2.2556
Low fat< 0.91g/100ml	0.6421	0.5361	0.7560	0.4344	0.2912	0.7340	1.9624	1.8105	2.2533
High fat > 6.01g/100ml	0.6701	0.5935	0.8085	0.3669	0.2453	0.4915	1.8241	1.7196	2.0467
Low protein < 3.04g/100ml	0.5964	0.4416	0.6803	0.3489	0.1851	0.4509	1.7851	1.5686	1.9969
High protein > 4.00g/100ml	0.8341	0.5821	**1.0299**	0.3941	0.1733	0.6418	1.9323	1.6175	2.2658
Milk with cefalexin at 4 µg/kg									
Normal milk = reference	2.0643	1.1942	2.2655	2.6033	2.4240	2.7619	0.7280	0.6340	0.8014
Low fat < 0.91g/100ml	2.0047	1.8124	2.3205	2.4090	1.9993	2.7775	0.6991	0.5902	0.7956
High fat > 6.01g/100ml	2.0045	1.8534	2.2464	2.3514	2.0715	2.6980	0.7291	0.6410	0.7939
Low protein < 3.04g/100ml	1.9130	1.5361	2.2154	2.1755	1.5775	2.5998	0.6979	0.5794	0.7914
High protein > 4.00g/100ml	2.2123	2.1022	2.3351	2.6035	2.4110	2.9853	0.8143	0.6680	0.9214

Notes: min: minimum; max: maximum. Tet: Tetracycline; Beta: β-lactam; Cefa: Cefalexin.

Discussion:
In general, the fat content and protein content had mostly no significant influence on the performance of the AnticFast® Beta-lactams & Tetracyclines & Cefalexin Rapid Test Kit result. No false positives were obtained with the blank milk. One milk sample with high protein content was found positive on the cefalexin channel (with ratios between 0.3672 and 0.4155). The sample also tested positive on other screening tests and LC-MS analysis showed the presence of cefalexin at 5.2 µg/l and cloxacillin at 0.22 µg/l (result not included in Table 13 or Figure 4). All positive samples were positive except for milk with a high protein content spiked with benzylpenicillin at 1 µg/kg and oxytetracycline at 4 µg/kg, with one borderline negative result (ratio 1.0299) on the tetracycline channel. This indicates a small 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 oxytetracycline: 100 µg/kg), it is expected not to have any detection problems at MRL.

6.4. Type of milk and animal origin influences

Methods and Materials:
Raw milk, UHT milk, sterilized milk, reconstituted milk powder and thawed milk were analysed in order to determine if the AnticFast® Beta-lactams & Tetracyclines & Cefalexin Rapid 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 & Cefalexin Rapid Test Kit is a suitable test for these types of milk coming from animal species other than the cow.

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;
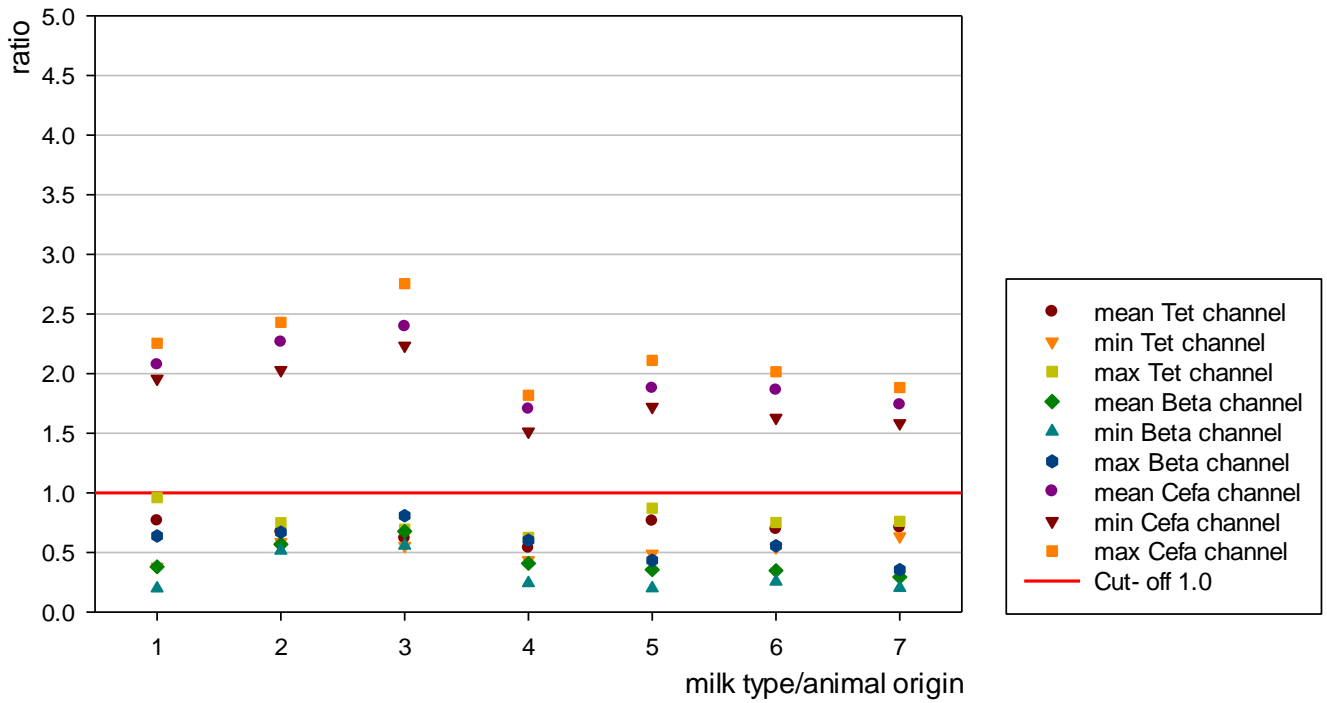 5 = Thawed milk;
  6 = Goats' milk;
  7 = Ewes' milk

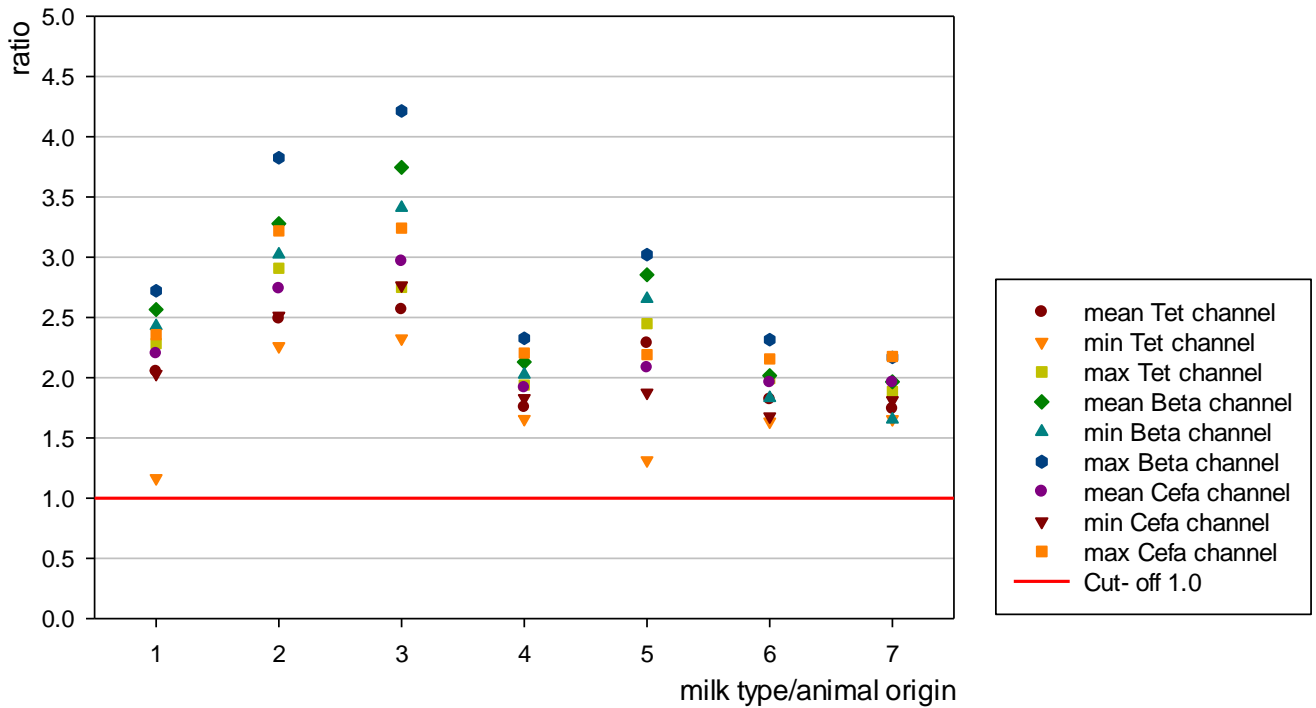Fig. 7. Results for blank milk, 10 replicates. Tet: Tetracycline; Beta: β-lactam; Cefa: Cefalexin.

Fig. 8. Results for milk spiked with benzylpenicillin at 1 µg/kg and oxytetracycline at 4 µg/kg, 10 replicates. Tet: Tetracycline; Beta: β-lactam; Cefa: Cefalexin.
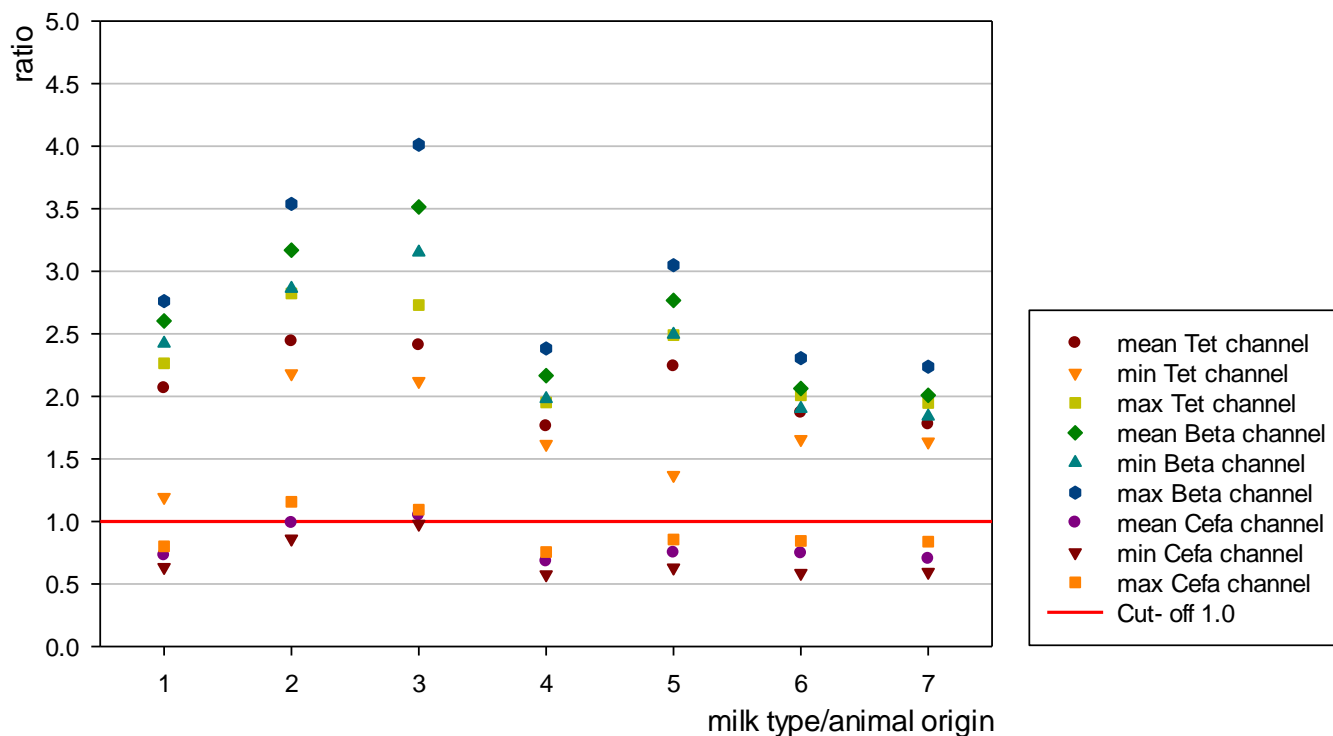
Fig. 9. Results for milk spiked with cefalexin at 4 µg/kg, 10 replicates. Tet: Tetracycline; Beta: β-lactam; Cefa: Cefalexin.

Discussion:

There could also be interest to use the AnticFast® Beta-lactams & Tetracyclines & Cefalexin Rapid 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 blank milk, all types of milk gave negative results. For milk types spiked with cefalexin at 4 µg/kg, UHT and sterilized milk samples gave some negative (borderline) results (4 and 8 negative results out of 10 spiked samples, respectively). Therefore, it can be concluded that an interference of detection occurs with heat treated milk. However, since the milk was spiked with a concentration far below MRL (100 µg/kg), no problems are expected for detection of cefalexin at MRL in heat treated milk. But no guarantee can be given without testing that a 95% detection capability will be observed in heat treated dairy products for compounds with a ccβ close to MRL in raw cows' milk .

For goats' and ewes' milk; blank samples were tested negative and spiked samples were tested positive.

The AnticFast® Beta-lactams & Tetracyclines & Cefalexin Rapid Test Kit can be used to analyse UHT milk, sterilized milk, thawed milk and reconstituted milk powder. And the test can also be used in ewes' and goats' milk.

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

	Ratio								
	Tetracycline channel			Beta-lactam channel			Cefalexin channel		
	mean	min	max	mean	min	max	mean	min	max
Blank milk									
normal raw cows' milk = reference	2.0507	1.1645	2.2831	2.5663	2.4298	2.7215	2.2003	2.0307	2.3583
UHT milk	2.4909	2.2623	2.9099	3.2796	3.0233	3.8266	2.7407	2.5145	3.2190
sterilized milk	2.5669	2.3269	2.7500	3.7464	3.4106	4.2144	2.9677	2.7666	3.2425
reconstituted milk powder	1.7529	1.6560	1.9384	2.1287	2.0247	2.3272	1.9161	1.8294	2.2048
thawed milk*	2.2879	1.3131	2.4497	2.8553	2.6547	3.0215	2.0821	1.8746	2.1919
goats' milk (blank n=20)	1.8199	1.6345	1.9921	2.0160	1.8305	2.3159	1.9591	1.6774	2.1565
ewes' milk (blank n=20)	1.7402	1.6555	1.8844	1.9643	1.6523	2.1695	1.9609	1.8122	2.1765
Milk with benzylpenicillin at 1 μg/kg + oxytetracycline at 4 μg/kg									
normal raw cows' milk = reference	0.7655	0.3812	0.9590	0.3787	0.1974	0.6381	2.0738	1.9558	2.2556
UHT milk	0.6694	0.5861	0.7487	0.5674	0.5146	0.6689	2.2653	2.0289	2.4310
sterilized milk	0.6182	0.5543	0.6956	0.6768	0.5568	0.8074	2.3958	2.2347	2.7543
reconstituted milk powder	0.5363	0.4357	0.6268	0.4083	0.2428	0.6023	1.7022	1.5131	1.8182
thawed milk	0.7630	0.4898	0.8705	0.3547	0.1992	0.4333	1.8771	1.7210	2.1106
goats' milk	0.6939	0.5416	0.7507	0.3485	0.2546	0.5544	1.8604	1.6289	2.0147
ewes' milk	0.7063	0.6347	0.7602	0.2939	0.2022	0.3555	1.7373	1.5832	1.8827
Milk with cefalexin at 4 μg/kg									
normal raw cows' milk = reference	2.0643	1.1942	2.2655	2.6033	2.4240	2.7619	0.7280	0.6340	0.8014
UHT milk	2.4406	2.1841	2.8242	3.1686	2.8628	3.5391	0.9873	0.8602	**1.1568**
sterilized milk	2.4089	2.1213	2.7301	3.5153	3.1518	4.0125	**1.0458**	0.9793	**1.0942**
reconstituted milk powder	1.7603	1.6174	1.9501	2.1662	1.9786	2.3842	0.6802	0.5743	0.7557
thawed milk	2.2409	1.3699	2.4914	2.7681	2.4950	3.0490	0.7491	0.6287	0.8556
goats' milk	1.8679	1.6567	2.0080	2.0618	1.9001	2.3058	0.7156	0.5864	0.8434
ewes' milk	1.7771	1.6363	1.9460	2.0083	1.8397	2.2382	0.7004	0.5957	0.8383

Notes: min: minimum; max: maximum.

6.5. Stability of reagents

6.5.1 Daily control samples

Methods and material:
The following control samples were analyzed daily with the AnticFast® Beta-lactams & Tetracyclines & Cefalexin Rapid 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 4 µg/kg
- Raw milk spiked with 4 µg/kg of cefalexin

Each day, also a negative and positive control (lotnumbers and expiry dates are equal to those of the kit reagents (lot 1 20201201G (expiration date 01/12/2021), lot 2 20201215G (expiration date 15/12/2021)) as provided in the kit were analysed. These controls were dissolved in 2 ml of HPLC water. The positive control contained 4 µg/kg of benzylpenicillin, 10 µg/kg of oxytetracycline and 15 µg/kg of cefalexin.

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

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

	Ratio											
Standard	**Tetracycline channel**				**Beta-lactam channel**				**Cefalexin channel**			
	mean	min	max	s_r	mean	min	max	s_r	mean	min	max	s_r
Blank milk												
	2.0799	1.6681	2.2985	0.1620	2.4085	1.3946	2.8661	0.37	2.2282	1.8394	2.4403	0.16
Milk spiked with benzylpenicillin at 1 µg/kg and oxytetracycline at 4 µg/kg												
	0.6698	0.5643	0.7764	0.06	0.2945	0.2158	0.4171	0.04	1.7287	1.3612	2.0652	0.17
Milk spiked with cefalexin at 4 µg/kg												
	2.1617	1.5116	2.3096	0.17	2.5898	1.2735	2.8236	0.37	0.6730	0.4875	0.9647	0.10
Controls included in the test kit												
Negative	1.7315	1.4644	1.8092	0.07	2.0515	1.9169	2.1697	0.07	1.8873	1.7700	2.0781	0.08
Positive	0.1784	0.1359	0.3301	0.04	0.1216	0.0990	0.1986	0.02	0.1118	0.0976	0.2124	0.02

Notes: 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 & Cefalexin Rapid 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 4 μg/kg of tetracycline and the milk samples spiked with a concentration of 4 μg/kg of cefalexin 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 of lot 3 20210909G (expiration date 09/09/2022) and lot 4 20211009G (expiration date 09/10/22) were used. For blank samples, the ratios are lower, while for samples doped with cefalexin at 4μg/kg, the ratios are closer to the cut-off on the cefalexin channel (less sensitive). As the concentration of cefalexin in de doped samples is very far below the MRL, there is no doubt that the compound will always be detected below its MRL.

The negative and positive controls inserted in the kit always gave correct results.
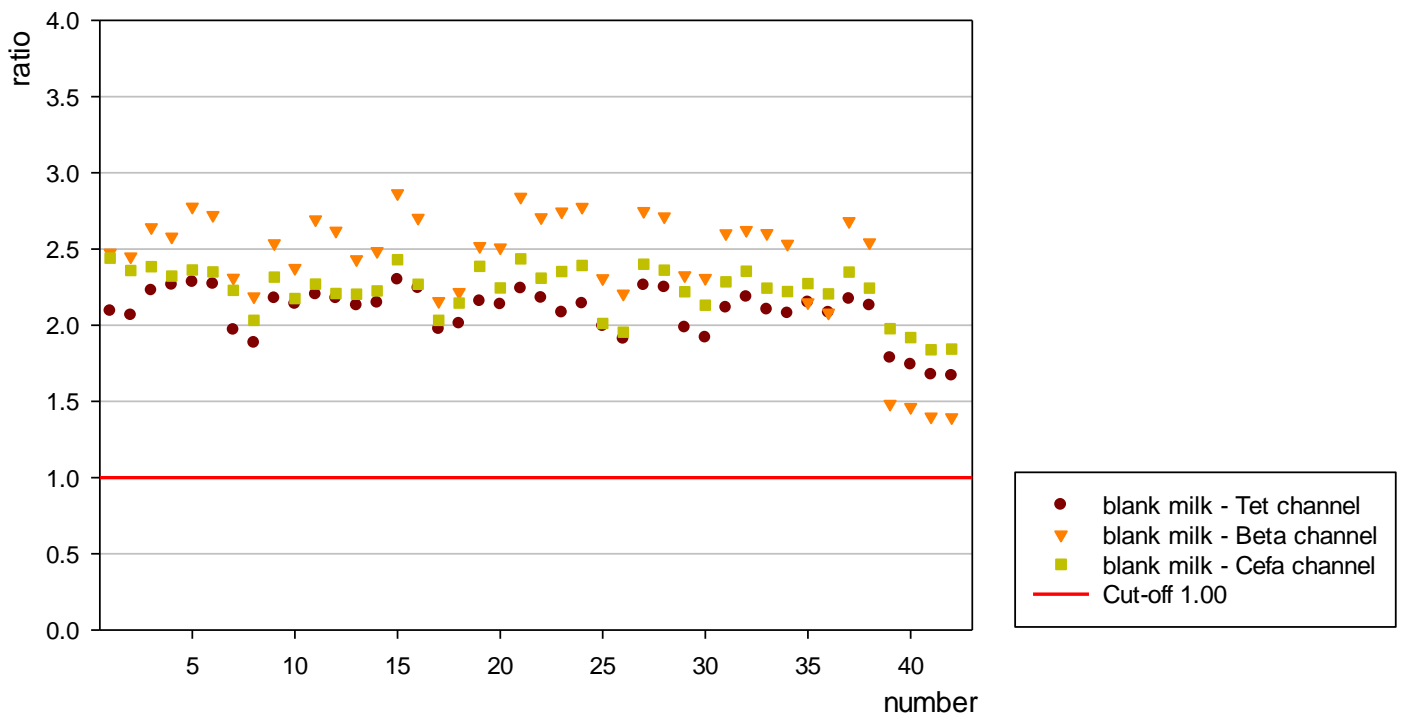
Fig. 10. AnticFast® Beta-lactams & Tetracyclines & Cefalexin Rapid Test Kit results (ratio) for the blank control samples. Tet: Tetracycline; Beta: β-lactam; Cefa: Cefalexin.
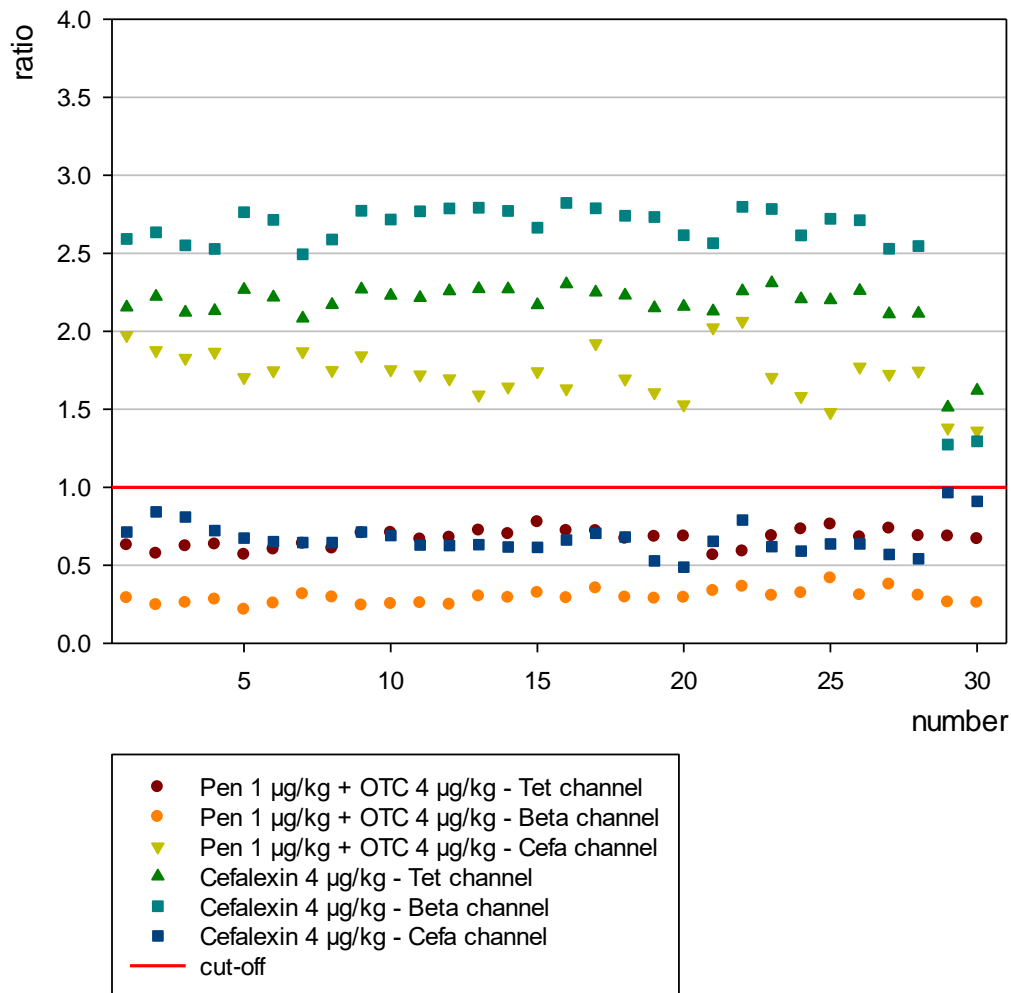
Fig. 11. AnticFast® Beta-lactams & Tetracyclines & Cefalexin Rapid Test Kit results (ratio) for the spiked control samples with benzylpenicillin at 1 µg/kg and oxytetracycline at 4 µg/kg and spiked with cefalexin at 4 µg/kg. Tet: Tetracycline; Beta: β-lactam; Cefa: Cefalexin.
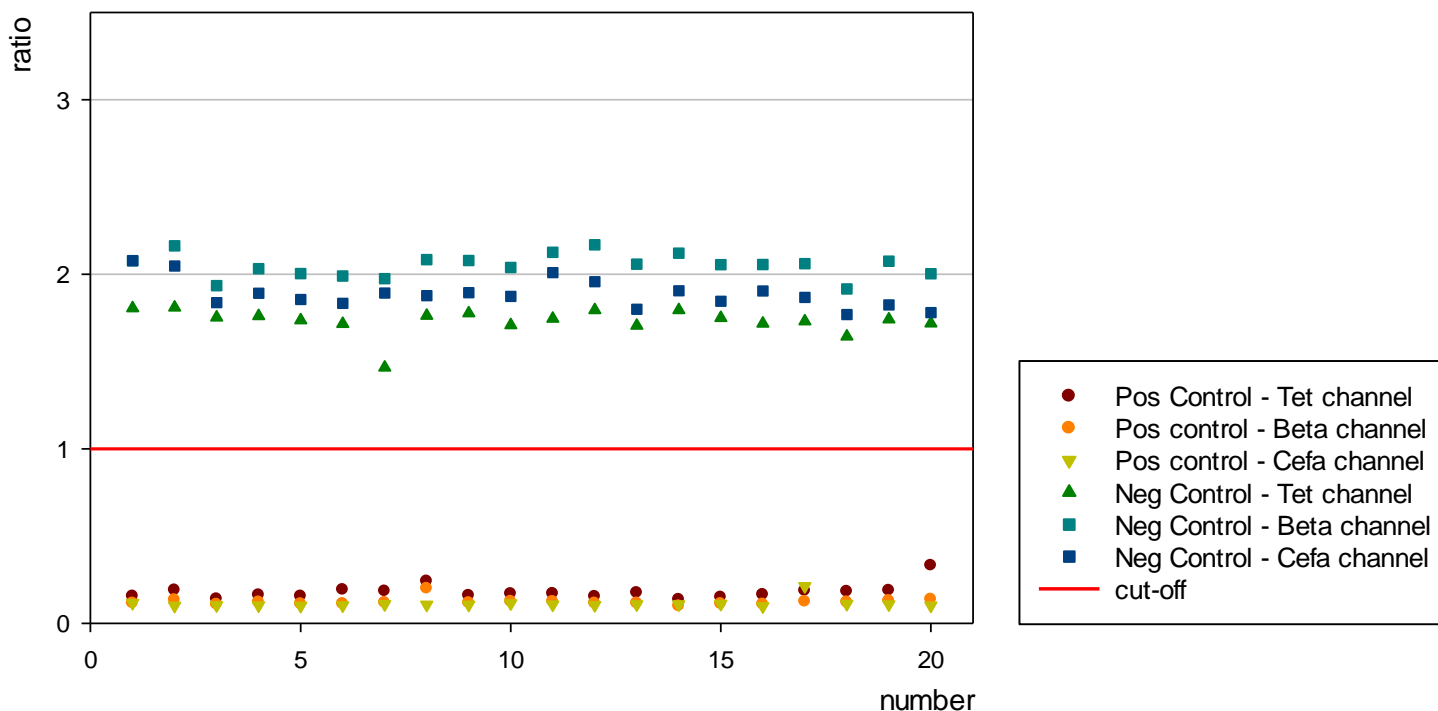
Fig. 12. AnticFast® Beta-lactams & Tetracyclines & Cefalexin Rapid Test Kit results (ratio) for the negative and positive controls inserted in the kit. Tet: Tetracycline; Beta: β-lactam; Cefa: Cefalexin.

6.5.2. Age of reagents

Methods and Materials:
AnticFast® Beta-lactams & Tetracyclines & Cefalexin Rapid Test Kit reagents (lot 20201201G with expiration date 01/12/2021) were used shortly after production date and 1 day before expiry date. Twenty raw blank cows' milk samples were tested, 20 doped milk samples with benzylpenicillin at 1 µg/kg and oxytetracycline at 4 µg/kg and 20 doped milk samples with cefalexin at 4 µg/kg.

Results:
For the stability of reagents regarding the age of reagents; the mean, the highest, and lowest reader values for each channel are given in Table 16.

Discussion:
Concerning the age of reagents, no significant changes are observed, blank samples were all negative and spiked samples were all positive. However, close to expiry date, it is noticed that the mean ratios of both blank samples are lower than close to production date. This was also noticed for spiked samples with cefalexin on the respective channel, while samples spiked with benzylpenicillin and oxytetracycline, showed higher mean ratio values when close to expiry date. For the latter, the variation in ratios is also smaller.

Table 16. Results of Anticfast® Beta-lactams & Tetracyclines & Cefalexin Rapid Test Kit stability of reagents (age of reagents).

Age of reagents	Ratio					
	Shortly after production			1 day before expiry date		
	Tet	Beta	Cefa	Tet	Beta	Cefa
Blank milk						
mean	2.0507	2.5663	2.2003	1.9546	2.3678	2.1797
min	1.1645	2.4298	2.0307	1.8724	2.2302	2.1018
max	2.2831	2.7215	2.3583	2.0067	2.4702	2.2603
Milk with benzylpenicillin at 1 µg/kg and oxytetracycline at 4 µg/kg						
mean	0.7655	0.3787	2.0738	0.8193	0.2133	1.8732
min	0.3812	0.1974	1.9558	0.7494	0.1583	1.6612
max	0.9590	0.6381	2.2556	0.8948	0.2636	1.9939
Milk with cefalexin at 4 µg/kg						
mean	2.0643	2.6033	0.7280	1.9529	2.3614	0.5374
min	1.1942	2.4240	0.6340	1.8790	2.2524	0.4336
max	2.2655	2.7619	0.8014	2.0723	2.4908	0.6721

Notes: min: minimum; max: maximum. Tet: Tetracycline; Beta: β-lactam; Cefa: Cefalexin.

7. Reliability of the instrumentation

No big technical problems were observed during validation.

Two small remarks for improvement:
- Consulting previous results on the reader is difficult as the ID code doesn't change automatically.
- The reader doesn't indicate invalid results when the control line is not formed well.

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 April 2021, AnticFast® Beta-lactams & Tetracyclines & Cefalexin Rapid Test Kit was integrated as rapid test in an interlaboratory study.

Results:

Identification of the samples:

Sample	Drug	Conc. (µg/kg)	MRL (µg/kg)
A	Sulfadoxine	100	100
B	Cefquinome	20	20
C	Cloxacillin	30	30
D	Benzylpenicillin	4	4
E	Chlortetracycline	100	100
F	Cefalexin	100	100
G	Blank	-	-
H	Ampicillin	4	4

Table 17. Results of Anticfast® Beta-lactams & Tetracyclines & Cefalexin Rapid Test Kit in the national ring trial of 22 April 2021 (Ooghe & Reybroeck, 2021).

LAB	Visual reading AnticFast® Beta-lactams & Tetracyclines & Cefalexin Rapid Test Kit								Instrumental reading (ratio + result) AnticFast® Beta-lactams & Tetracyclines & Cefalexin Rapid 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																	
ILVO	-	-	-	-	**+**	-	-	-	1.8818 NEG	2.0304 NEG	1.9770 NEG	2.2433 NEG	**0.1682 POS**	2.2204 NEG	2.1337 NEG	2.0190 NEG	20201201G 1/12/2021 BMZ6000 Portable Strip Reader
CEFALEXIN																	
ILVO	-	-	-	-	-	**+**	-	-	2.0445 NEG	1.9706 NEG	1.9100 NEG	1.8363 NEG	2.0305 NEG	**0.2223 POS**	2.1570 NEG	1.9233 NEG	
BETA-LACTAM																	
ILVO	-	**+**	**+**	**+**	-	-	-	**+**	2.1755 NEG	**0.2505 POS**	**0.1334 POS**	**0.1914 POS**	2.4234 NEG	2.7376 NEG	2.6302 NEG	**0.2785 POS**	

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.

Conclusion:
Good results were obtained with AnticFast® Beta-lactams & Tetracyclines & Cefalexin Rapid Test Kit.

All milk samples fortified with ß-lactam antibiotics (samples B, C, D, F & H) were screened positive on the β-lactam test line of AnticFast® Beta-lactams & Tetracyclines & Cefalexin Rapid Test Kit, except for sample F (100 ppb cefalexin), which was screened positive on the cefalexin test line.
The milk sample spiked with 100 ppb chlortetracycline (sample E) was screened positive on the tetracycline test line of AnticFast® Beta-lactams & Tetracyclines & Cefalexin Rapid Test Kit.
Hence, in this ring test cefquinome, cloxacillin, benzylpenicillin, chlortetracycline, cefalexin and ampicillin are detected at MRL with AnticFast® Beta-lactams & Tetracyclines & Cefalexin Rapid Test Kit.

Negative results were obtained for the blank milk (sample G) on all channels and for the milk samples spiked 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 & Cefalexin Rapid Test Kit.

9. Final conclusion

Results of this validation show that the AnticFast® Beta-lactams & Tetracyclines & Cefalexin Rapid 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 20210909G (expiration date 09/09/2022) and lot 20211009G (expiration date 09/10/22)), all β-lactams, including cefalexin can be detected at least in 95% of the replicates at their respective MRL except for desfuroylceftiofur. The detection capability of desfuroylceftiofur was not determined, but was above MRL. All tetracyclines and their 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.
The test could also be used to screen other milk types or milk coming from animal species other than the cow, on the presence of residues of β-lactams, tetracyclines and cefalexin.
The test is suitable for other types of milk, such as UHT milk, sterilized milk, thawed milk and reconstituted milk powder. For UHT and sterilized milk, one should take notice that some hampering of detection is possible, leading to a lower sensitivity for some compounds then those described in Table 4. AnticFast® Beta-lactams & Tetracyclines & Cefalexin Rapid Test Kit could also be used to screen ewes' and goats' milk.

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