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***Validation report AnticFast® Beta-
lactams & Tetracyclines &
Sulfonamides Rapid Test Kit***
(Order n° JC0458)
(Meizheng Bio-Tech China)

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1. Introduction

AnticFast® Beta-lactams & Tetracyclines & Sulfonamides Rapid Test Kit (Meizheng Bio-Tech, China) is a qualitative two-step (2 min + 5min) rapid lateral flow assay to detect β -lactam (penicillins and cephalosporins), tetracycline antibiotic and sulfonamide 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 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 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)). With these improved reagents also some extra blank farm and tanker milk samples were tested. All other validation (initial report) was performed using reagents lot 1: 20200918G (expiration date 18/09/2021) and lot 2: 20200821G (expiration date 21/08/2021).

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). 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 μ L 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 2 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 & Sulfonamides Rapid Test Kit test strip

The configuration of the AnticFast® Beta-lactams & Tetracyclines & Sulfonamides Rapid Test Kit test strip is shown in Figure 1.

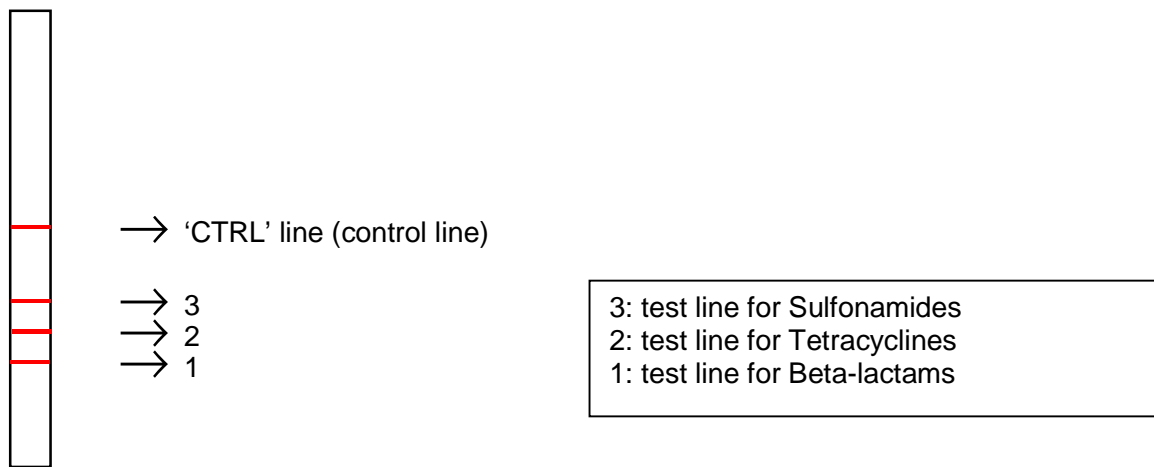


Fig. 1. Configuration of an AnticFast® Beta-lactams & Tetracyclines & Sulfonamides Rapid Test Kit test strip.

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 & Sulfonamides 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 & Sulfonamides 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.

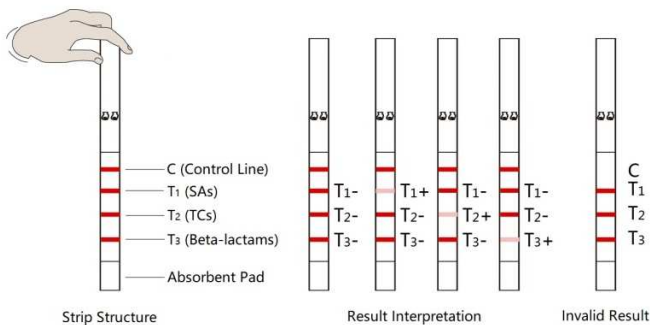


Fig. 3. Visual interpretation of the color formation on the test strips of AnticFast® Beta-lactams & Tetracyclines & Sulfonamides Rapid Test Kit (Anon.; 2020).

3. Detection capability

Methods and Materials:

Spiking of antibiotic-free (blank) raw milk with β -lactams (penicillins and cephalosporins), tetracyclines and sulfonamides.

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 & Sulfonamides Rapid Test Kit was determined for all different compounds belonging to the β -lactam, tetracycline and sulfonamide 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 $\geq 0.9 - \leq 1.0$ 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 $\mu\text{g}/\text{kg}$)	Increment (in $\mu\text{g}/\text{kg}$)
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 1.5 µg/kg and oxytetracycline at 7 µg/kg - twice
- blank raw milk spiked with sulfadoxine at 3 µg/kg – twice

Detection capability tests were performed with 4 different lot numbers of reagents: lot 1 20200918G (expiration date 18/09/2021) and lot 2 20200821G (expiration date 21/08/2021), 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
Amoxicillin trihydrate	Sigma Aldrich	31586	BCCB1309
Ampicillin trihydrate	Sigma Aldrich	31591	BCCD7541
Cefacetile	Sigma Aldrich	C231500	2-MAX-147-3
Cefalonium hydrate	Sigma Aldrich	32904	BCBV1595
Cefapirin sodium	Sigma Aldrich	43989	BCCC5208
Cefazolin (European Pharmacopoeia Reference)	Sigma Aldrich	C0682800	5.0
Cefoperazone dihydrate	Sigma Aldrich	32426	BCBX0019
Cefquinome sulfate	Sigma Aldrich	32472	BCBW2550
Cefquinome sulfate	LGC Standards	C16998175	G1005777
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
Cloxacilline sodium salt monohydrate VETRANAL	Sigma Aldrich	46140	BCBW1061
Colistin sulfate	Sigma Aldrich	C4461	049M4836V
Dapsone	Sigma Aldrich	46158	BCBX0187
Desacetyl cephalirin sodium salt	LGC standards	682120	799058
Desfuroyl ceftiofur	TRC	D289980	5-WBZ-57-5
Dicloxacillin	Sigma Aldrich	46182	BCBX4662
Doxycycline Hyclate VETRANAL	Sigma Aldrich	33429	BCBS7684V
Enrofloxacin	Sigma Aldrich	33699	BCBZ6597
Erythromycin A dihydrate	Sigma Aldrich	46256	BCBS7769V

Table 3 continued. Standard material used in this validations study.

Lincomycin Hydrochloride 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
Sulfamethazine	Sigma Aldrich	46802	SZBG035XV
Sulfamerazine	Sigma Aldrich	46826	BCBS3635V
Sulfadiazine	Sigma Aldrich	S8626	056M4795V
Sulfadiazine sodium salt	Sigma Aldrich	S6387	MKCJ7742
Sulfamonomethoxine	Sigma Aldrich	32091	BCBW4869
Sulfadimethoxine	Sigma Aldrich	46794	BCBX1159
Sulfadoxine	Sigma Aldrich	S7821	SLBL5136V
Sulfametoxydiazine/ sulfameter	Sigma Aldrich	46834	BCBW1230
Sulfisomidine	Sigma Aldrich	46908	BCBS3734V
Sulfachloropyridazine	Sigma Aldrich	46778	BCBT3237
Sulfamethoxy pyridazine	Sigma Aldrich	46858	BCCB5494
Sulfabenzamide	Sigma Aldrich	46762	BCBW2565
Sulphaquinoxaline	Sigma Aldrich	45662	BCBV0374
Sulfamethoxazole/ Benzenesulfonamide	Sigma Aldrich	31737	BCBR8039V
Sulfamethoxypyrazine	LGC Standards	S699230	3-EOD-153-1
Sulfamoxole/ sulfamethoxydazine	Sigma Aldrich	46866	BCBT9181
Sulfaclozine	Sigma Aldrich	32421	BCBX5024
Sulfisoxazole	Sigma Aldrich	31739	BCBT2201
Sulfamethizole	Sigma Aldrich	46842	BCBT9838
Sulfaethoxy pyridazine	Sigma Aldrich	02743	BCCC4439
Tetracycline hydrochloride	Sigma Aldrich	31741	BCCC9767
Trimethoprim, minimum 98%TLC	Sigma Aldrich	46984	BCBX0881
4-epimer chlortetracycline	Acros Organics	268231000	A0406408
4-epimer oxytetracycline	Acros Organics	257711000	A0395560
4-epimer tetracycline	Acros Organics	233121000	A0397675

Results:

A summary of the ILVO detection capabilities is given in Table 4.

Table 4. Detection capability (in µg/kg) of AnticFast® Beta-lactams & Tetracyclines & Sulfonamides 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	2	
ampicillin	4	3	
amoxicillin	4	4	
oxacillin	30	7	
cloxacillin	30	7	
dicloxacillin	30	5	
nafcillin	30	25	
Cefalosporins			
ceftiofur	100 ^a		60
desfuroylceftiofur	100 ^a	-- (>MRL) *	
cefquinome	20	20	
cefazolin	50		30
cephapirin	60 ^b	16	
desacetylcephapirin	60 ^b	50	
cefacetrile	125	60	
cefoperazone	50	3	
cefalexin	100	-- (>MRL) *	
cefalonium	20	2	
Tetracyclines			
tetracycline	100 ^c	6	
4-epimer of tetracycline	100 ^c	12	
oxytetracycline	100 ^c	6	
4-epimer of oxytetracycline	100 ^c	10	
chlortetracycline	100 ^c	20	
4-epimer of chlortetracycline	100 ^c	50	
doxycycline	-- ^d	2	
Sulfonamides			
sulfamethazine	100 ^e	20	
sulfamerazine	100 ^e	5	
sulfadiazine	100 ^e	3	
sulfamonomethoxine	100 ^e	2	
sulfadimethoxine	100 ^e	3	

Table 4 continued.

Antibiotic Group/ antibiotic	EU MRL (µg/kg)	Detection capability (µg/kg)	
		Lot 1 & 2	Lot 3 & 4
sulfadoxine	100 ^e	3	
sulfametoxydiazine	100 ^e	3	
sulfisomidine	100 ^e	4	
sulfachlorpyridazine	100 ^e	5	
sulfamethoxy pyridazine	100 ^e	25	
sulfabenzamide	100 ^e	10	
sulphaquinoxaline	100 ^e	25	
sulfamethoxazole/ benzenesulfonamide	100 ^e	60	
sulfamethoxypyrazine	100 ^e	4	
sulfamoxole/ sulfamethoxydiazine	100 ^e	18	
sulfaclozine	100 ^e	6	
sulfisoxazole	100 ^e	45	
sulfamethizole	100 ^e	4	
sulfaethoxy pyridazine	100 ^e	45	

Notes: lot 1 20200918G (expiration date 18/09/2021), lot 2 20200821G (expiration date 21/08/2021), 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. 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,

^e: The combined total residues of all substances within the sulfonamide group should not exceed 100 µg/kg.

Discussion:

The AnticFast® Beta-lactams & Tetracyclines & Sulfonamides Rapid Test Kit is capable to detect the residues of β-lactams (penicillins and cephalosporins), tetracyclines and sulfonamides 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 cefalexin and desfuroylceftiofur. The 95% detection capability of these two compounds was not determined as these could not be detected at MRL and it was therefore not requested by the kit manufacturer.

It's worth noting that ceftiofur and cefazolin 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 CC β of 60 $\mu\text{g}/\text{kg}$ was determined for ceftiofur and of 30 for cefazolin. The impact of the improvement of the reagents on the CC β for the other β -lactams and other families was not determined.

All tetracyclines, including 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 $\mu\text{g}/\text{kg}$ on.

All tested sulfonamides were detected at least in 95% of the replicates at their respective MRL.

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 & Sulfonamides Rapid Test Kit was tested by analysing milk spiked with β -lactam, tetracycline and sulfonamide 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, tetracycline and sulfonamide test line. Raw milk was spiked at a high concentration (100xMRL or 100xMRPL 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) 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.

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 2,000 µg/kg on.

AnticFast® Beta-lactams & Tetracyclines & Sulfonamides Rapid Test Kit is a highly specific test for detection of β-lactams, tetracycline and sulfonamides in milk and does not detect compounds from the 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 & Cefalexin Rapid Test Kit

Family	Compound	MR(P)L (µg/kg)	Conc. spiked in milk (µg/kg)	Sulfa channel		Tetra channel		Beta channel	
				Ratio	Result	Ratio	Result	Ratio	Result
Penicillins	Benzylpenicillin	4	400	2.6625	-	3.1115	-	0.3146	+
Cephalosporins	Cefalonium	20	2,000	2.7792	-	3.2832	-	0.3650	+
Tetracyclines	Oxytetracycline	100 ^a	10,000	2.7757	-	0.1844	+	4.1502	-
Sulfonamides	Sulfadiazine	100 ^b	10,000	0.2918	+	2.8308	-	3.7433	-
Aminoglycosides	Neomycin B	1,500	150,000	2.5329	-	3.0136	-	3.5014	-
Macrolides	Erythromycin	40	4,000	2.5796	-	2.5977	-	3.3870	-
Quinolones	Enrofloxacin	100 ^c	10,000	2.9065	-	3.5198	-	4.4430	-
Amphenicols	Chloramphenicol	0.3 ^d	30	2.7768	-	3.5708	-	4.7349	-
Polymyxins	Colistin	50	5,000	2.9106	-	3.5814	-	4.5965	-
Lincosamides	Lincomycin	150	15,000	2.7157	-	3.1298	-	4.0414	-
β-lactamase inhibitors	Clavulanic acid	200	20,000	2.7438	-	2.8421	-	0.2936	+
Diamino pyrimidine derivatives	Trimethoprim	50	5,000	2.9682	-	3.5973	-	4.6220	-
Others	Dapsone	5 ^e	500	2.7856	-	3.6859	-	4.9637	-

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; Sulfa: sulfonamides, Tetra: tetracycline; Beta: β-lactam.

^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 sulfonamide 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:

300 farm and 301 tanker load milk samples were tested with AnticFast® Beta-lactams & Tetracyclines & Sulfonamides Rapid Test Kit (lot 1 20200918G (expiration date 18/09/2021), lot 2 20200821G (expiration date 21/08/2021)) and other microbiological and receptor screening tests. With the improved reagents of lot 3 20210909G (expiration date 09/09/2022) and lot 4 20211009G (expiration date 09/10/22), 48 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.

Table 6a. AnticFast® Beta-lactams & Tetracyclines & Sulfonamides Rapid Test Kit results for blank farm and tanker milk samples (reagents lot 1 and 2).

Lot 1 and 2	Blank farm milk (n=300)			Blank tanker milk (n=300)		
	Ratio			Ratio		
	Sulfa	Tetra	Beta	Sulfa	Tetra	Beta
Mean	2.8745	3.2288	4.2444	2.9339	3.2912	4.3760
Min	1.6889	0.3385	1.7015	1.5703	1.9341	1.3059
Max	3.3982	3.8158	5.2755	3.5533	4.0737	5.4999
s_r	0.21	0.26	0.38	0.22	0.26	0.36
CV%	7.22	8.18	8.87	7.52	7.75	8.29

Notes: mean: mean ratio; min: lowest ratio; max: highest ratio; Sulfa: sulfonamides, Tetra: tetracycline; Beta: β -lactam. s_r; Standard Deviation; CV(%): Relative Standard Deviation.

Results and discussion:

Lot 1 and 2: Of the 300 farm milk samples and 301 tanker milk samples, all tested negative for β -lactams and sulfonamides on AnticFast® Beta-lactams & Tetracyclines & Sulfonamides Rapid Test Kit, except for one farm milk samples which tested positive on the tetracycline channel (ratio: 0.3385). It was however noticed that the ratios of all channels were significantly lower than otherwise. Repetition of the sample gave twice negative results.

Also one tanker load milk samples tested positive on the tetracycline channel (ratio: 0.5074). Repetition of the sample gave positive results (ratios: 0.4073 and 0.4164). Testing with other screening tests showed that this sample was a real positive, so this sample was not incorporated in Table 6a.

Giving these results, it is concluded that in total no false positive results were obtained upon 600 samples on the beta-lactam and sulfonamide channel. And one false positive result (=0.002%) is obtained on 600 samples on the tetracycline channel. The results are summarized in Table 6a.

For the improved reagents of lot 3 and 4: Of the 48 farm milk and 49 tanker load milk samples, all tested negative for β -lactams, tetracyclines and sulfonamides on AnticFast® Beta-lactams & Tetracyclines & Sulfonamides Rapid Test Kit. Giving these results, it is concluded that in

total no false positive results were obtained upon 97 samples on all test channels. The results are summarized in Table 6b.

Table 6b. AnticFast® Beta-lactams & Tetracyclines & Sulfonamides Rapid Test Kit results for blank farm and tanker milk samples (reagents lot 3 and 4).

Lot 3 and 4	Farm milk (n=48)			Tanker milk (n=49)		
	Ratio			Ratio		
	Sulfa	Tetra	Beta	Sulfa	Tetra	Beta
Mean	1.5751	1.7810	1.7171	1.5418	1.7469	1.7324
Min	1.3822	1.5505	1.3593	1.3242	1.5455	1.4702
Max	1.7875	1.9860	1.9598	1.7140	1.9675	1.9416
s _r	0.09	0.09	0.12	0.08	0.09	0.10
CV%	5.59	5.08	6.71	5.29	4.95	5.70

Notes: mean: mean ratio; min: lowest ratio; max: highest ratio; Sulfa: sulfonamides, Tetra: tetracycline; Beta: β-lactam. 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 & Sulfonamides 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	Sulfonamides			Tetracyclines			Beta-lactams		
	Mean	s _r	CV%	Mean	s _r	CV%	Mean	s _r	CV%
Blank milk	2.8811	0.05	1.68	3.3108	0.04	1.30	4.2201	0.05	1.10
Low positive milk	0.8800	0.00	0.39	0.8417	0.00	0.49	0.9042	0.01	1.19
High positive milk	0.4878	0.00	0.45	0.2803	0.01	3.55	0.3764	0.01	1.65

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 3.55%).

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 & Sulfonamides 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	Sulfonamides			Tetracyclines			Beta-lactams		
	Mean	s _r	CV%	Mean	s _r	CV%	Mean	s _r	CV%
Blank milk	2.8586	0.17	6.07	3.4764	0.19	5.48	4.4906	0.22	5.00
Low positive milk	0.8981	0.04	4.45	0.8884	0.05	5.98	0.8623	0.06	7.40
High positive milk	0.4448	0.02	4.10	0.4004	0.03	7.37	0.3764	0.01	1.65

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

Discussion:

The repeatability of the test was also good, low standard deviation values were obtained. The highest variance value is 7.40%.

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 2+5 minutes.

Methods and Materials:

Blank and spiked milk samples containing benzylpenicillin at 1.5 µg/kg and oxytetracycline at 7 µg/kg or spiked milk containing sulfadoxine at 3 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 & Sulfonamides 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 all positive results stayed positive. Deviating the length of the incubation from the standard 2' + 5', is giving less variation in ratios for spiked milk and in general slightly more positive results on all channels.

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

Length of incubation steps	Ratio								
	Blank milk			Milk spiked with benzylpenicillin at 1.5 µg/kg and oxytetracycline at 7 µg/kg			Milk spiked with sulfadoxine at 3 µg/kg		
	Sulfa	Tetra	Beta	Sulfa	Tetra	Beta	Sulfa	Tetra	Beta
1 minutes 45 seconds + 4 minutes 30 seconds									
mean	3.0754	3.2634	4.1671	2.9186	0.6292	0.4961	0.6836	2.9762	3.8228
min	2.8749	3.0575	3.9407	2.7906	0.5688	0.4712	0.5214	2.8344	3.6525
max	3.2580	3.5535	4.3022	3.0394	0.7214	0.5244	0.9035	3.1460	4.0013
1 minutes 45 seconds + 5 minutes									
mean	2.9905	3.1793	4.0625	2.8298	0.5765	0.4947	0.5495	2.9240	3.6935
min	2.9239	3.1339	3.8883	2.7473	0.5514	0.4612	0.4913	2.7449	3.6450
max	3.0789	3.2082	4.1978	2.9501	0.5942	0.5333	0.5950	3.2258	3.7808
1 minutes 45 seconds + 5 minutes 30 seconds									
mean	2.8920	3.2027	4.0220	2.8378	0.6238	0.4762	0.5129	2.8776	3.5226
min	2.8224	3.1889	3.6926	2.6560	0.5230	0.3706	0.4715	2.7570	3.4251
max	2.9689	3.2240	4.2338	3.0640	0.7294	0.5611	0.5377	3.1004	3.6564
2 minutes + 4 minutes 30 seconds									
mean	3.0027	3.2925	4.2405	2.8663	0.6161	0.4815	0.6246	3.0248	3.9298
min	2.8227	3.0913	4.0636	2.8516	0.6078	0.4549	0.5666	2.9194	3.8553
max	3.1449	3.4048	4.4141	2.8739	0.6248	0.4949	0.6684	3.2021	3.9693
2 minutes + 5 minutes (REF)									
mean	3.0564	3.2917	4.2068	3.1042	0.6743	0.5710	0.6647	2.9983	3.7974
min	2.9984	3.1834	3.6767	2.8934	0.6033	0.4705	0.5564	2.9584	3.6186
max	3.1525	3.4322	4.5707	3.2451	0.8042	0.7240	0.8389	3.0770	3.9226

2 minutes + 5 minutes 30 seconds									
mean	2.9479	3.2149	3.9492	2.7579	0.5488	0.3901	0.5360	2.8012	3.5101
min	2.7721	3.0224	3.8225	2.6846	0.5114	0.3782	0.4726	2.7447	3.2839
max	3.2244	3.5871	4.1256	2.9034	0.5686	0.4078	0.6600	2.9080	3.6669
2 minutes 15 seconds + 4 minutes 30 seconds									
mean	2.9830	3.3500	4.1727	2.6439	0.5631	0.4011	0.5716	2.8603	3.6721
min	2.9379	3.2806	3.8993	2.6110	0.5340	0.3915	0.4918	2.6907	3.5567
max	3.0721	3.3870	4.4599	2.6778	0.6003	0.4136	0.6350	3.0241	3.7699
2 minutes 15 seconds + 5 minutes									
mean	2.8389	3.1806	4.0282	2.8581	0.6432	0.4550	0.5699	3.0954	3.8265
min	2.7172	3.1620	3.6859	2.7758	0.5851	0.4045	0.5444	2.9750	3.5049
max	2.9645	3.2127	4.2195	2.9309	0.6917	0.5541	0.5890	3.2316	4.1945
2 minutes 15 seconds + 5 minutes 30 seconds									
mean	2.5315	3.1321	3.8405	2.7742	0.6314	0.3697	0.5361	2.8023	3.5225
min	1.7717	3.0103	3.5826	2.6554	0.6072	0.3635	0.4380	2.6430	3.1629
max	2.9751	3.2249	3.9838	2.8400	0.6547	0.3783	0.6316	2.9499	3.8592

Notes: REF: reference; mean: mean ratio; min: minimum ratio; max: maximum ratio; Sulfa: sulfonamides, Tetra: tetracycline; Beta: β -lactam.

6.1.2. Delay of reading

Methods and Materials:

Blank and spiked milk samples containing benzylpenicillin at 1.5 $\mu\text{g}/\text{kg}$ and oxytetracycline at 7 $\mu\text{g}/\text{kg}$ or spiked milk containing sulfadoxine at 3 $\mu\text{g}/\text{kg}$ (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.

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. A small increase of ratio (less positive) is observed for spiked milk on the beta-lactam and tetracycline channel with increasing delay of reading.

Table 10. Ratios obtained when testing blank and spiked milk samples and reading the AnticFast® Beta-lactams & Tetracyclines & Sulfonamides 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		
	Sulfa	Tetra	Beta	Sulfa	Tetra	Beta	Sulfa	Tetra	Beta
Blank milk									
mean	2.9465	3.2723	3.9182	2.7304	3.1746	3.8337	2.4734	2.9692	3.5854
min	2.8913	3.2188	3.7694	2.6808	3.0401	3.7001	2.4477	2.7910	3.4696
max	3.0421	3.3648	4.1098	2.7560	3.3634	3.9755	2.5178	3.1765	3.7158
Milk spiked with benzylpenicillin at 1.5 µg/kg and oxytetracycline at 7 µg/kg									
mean	2.8315	0.6187	0.4492	2.7886	0.6443	0.4801	2.6792	0.7262	0.5933
min	2.5422	0.5614	0.3941	2.5663	0.5633	0.4062	2.5247	0.5528	0.5154
max	3.1232	0.6723	0.5217	3.1147	0.7225	0.5856	2.8531	0.9041	0.6370
Milk spiked with sulfadoxine at 3 µg/kg									
mean	0.5534	3.0432	3.6095	0.5362	3.0987	3.6139	0.5306	3.0590	3.5619
min	0.4728	2.8317	3.3234	0.4537	2.8609	3.2711	0.4188	2.9605	3.3816
max	0.6149	3.1699	4.0331	0.6037	3.2569	4.1086	0.6282	3.1834	3.8264

Notes: REF: reference; mean: mean ratio; min: minimum ratio; max: maximum ratio; Sulfa: sulfonamides, Tetra: tetracycline; Beta: β-lactam.

6.1.3. Volume of the milk

Methods and Materials:

Blank and spiked milk samples containing benzylpenicillin at 1.5 µg/kg and oxytetracycline at 7 µg/kg or spiked milk containing sulfadoxine at 3 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 220µl 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 & Sulfonamides Rapid Test Kit.

Volume of milk	Ratio								
	180 µl			200 µl (REF)			220 µl		
	Sulfa	Tetra	Beta	Sulfa	Tetra	Beta	Sulfa	Tetra	Beta
Blank milk									
mean	2.9606	3.3329	4.2302	3.0564	3.2917	4.2068	3.0588	3.2930	4.0235
min	2.8957	3.2772	4.1657	2.9984	3.1834	3.6767	2.8743	3.1338	3.7777
max	3.0315	3.4440	4.3191	3.1525	3.4322	4.5707	3.2965	3.5335	4.4076
Milk spiked with benzylpenicillin at 1.5 µg/kg and oxytetracycline at 7µg/kg									
mean	2.8337	0.6858	0.5658	3.1042	0.6743	0.5710	2.7770	0.6009	0.3620
min	2.7532	0.6468	0.5522	2.8934	0.6033	0.4705	2.6944	0.5587	0.3192
max	2.8762	0.7068	0.5818	3.2451	0.8042	0.7240	2.8470	0.6268	0.3878
Milk spiked with sulfadoxine at 3 µg/kg									
mean	0.5865	3.0716	3.7234	0.6647	2.9983	3.7974	0.5506	2.9404	3.6159
min	0.5555	3.0064	3.3826	0.5564	2.9584	3.6186	0.4996	2.8288	3.3167
max	0.6072	3.1476	3.9000	0.8389	3.0770	3.9226	0.5777	3.1128	3.7947

Notes: REF: reference; mean: mean ratio; min: minimum ratio; max: maximum ratio; Sulfa: sulfonamides, Tetra: tetracycline; Beta: β-lactam.

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 & Sulfonamides Rapid Test Kit result. Besides blank milk also spiked milk samples containing benzylpenicillin at 1.5 µg/kg and oxytetracycline at 7 µg/kg or spiked milk containing sulfadoxine at 3 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 & Sulfonamides 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 (more positive) ratios were obtained for all channels and a smaller range of variation for spiked milk was noticed on the sulfonamide channel.

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

Milk temperature	Ratio					
	1-4°C (REF)			20°C		
	Sulfa	Tetra	Beta	Sulfa	Tetra	Beta
Blank milk						
mean	3.0564	3.2917	4.2068	2.8744	3.0285	3.7154
min	2.9984	3.1834	3.6767	2.6952	2.9031	3.5053
max	3.1525	3.4322	4.5707	2.9727	3.1612	3.8756
Milk with benzylpenicillin at 1.5 µg/kg and oxytetracycline at 7 µg/kg						
mean	3.1042	0.6743	0.5710	2.8347	0.5818	0.4027
min	2.8934	0.6033	0.4705	2.6257	0.4845	0.3571
max	3.2451	0.8042	0.7240	3.0126	0.6857	0.4536
Milk with sulfadoxine at 3 µg/kg						
mean	0.6647	2.9983	3.7974	0.5316	2.9808	3.4240
min	0.5564	2.9584	3.6186	0.5023	2.8371	3.3671
max	0.8389	3.0770	3.9226	0.5604	3.1195	3.5320

Notes: REF: reference; mean: mean ratio; min: minimum ratio; max: maximum ratio. Sulfa: sulfonamides, Tetra: tetracycline; Beta: β-lactam.

6.3. Milk composition and milk type influences

Methods and Materials:

Total bacterial count

Normal milk samples and milk samples with a high total bacterial count (TBC >1.4 ×10⁶ CFU per ml) were analysed and the ratios obtained were compared in order to study the impact of the total bacterial count on the AnticFast® Beta-lactams & Tetracyclines & Sulfonamides Rapid Test Kit result. The milk samples with a high total bacterial count were obtained by keeping normal milk samples during 4-6 hours at room temperature. The final bacterial count was determined by performing a spiral plate count (Eddy Jet. IUL sa. Barcelona. Spain) on Plate count agar plates after 3 days incubation at 30°C.

Fat content

Normal milk samples and milk samples with a low (<1.40 g per 100 g) 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 & Sulfonamides 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 with a MilcoScan 4000 and the composition internally at ILVO checked with a Lactoscope FT-A.

Protein content

Normal milk samples and milk samples with a low (<2.99 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 & Sulfonamides 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 and the composition internally at ILVO checked 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 = High total bacterial count (TBC >1.4×10⁶ CFU/ml)
- 3 = Low fat content (<1.40 g/100 g);
- 4 = Low protein (<2.99 g/100 ml).

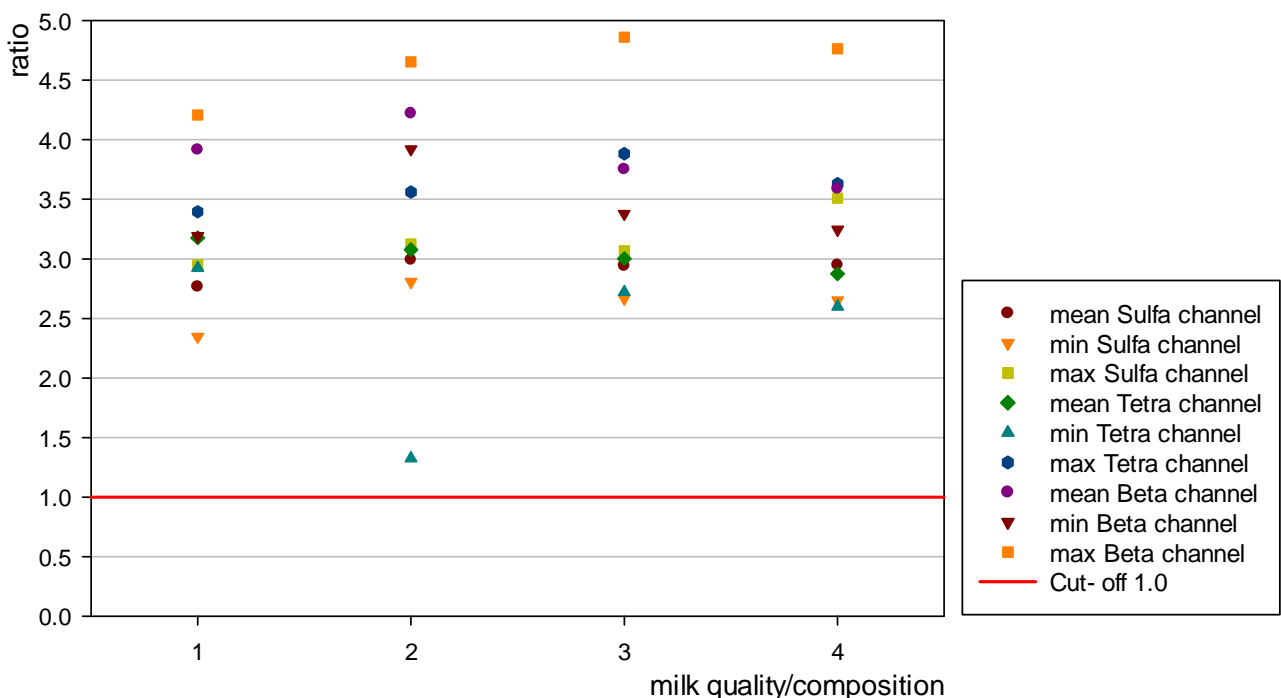


Fig. 4. Results for blank milk, 10 replicates. Sulfa: sulfonamides, Tetra: tetracycline; Beta: β-lactam.

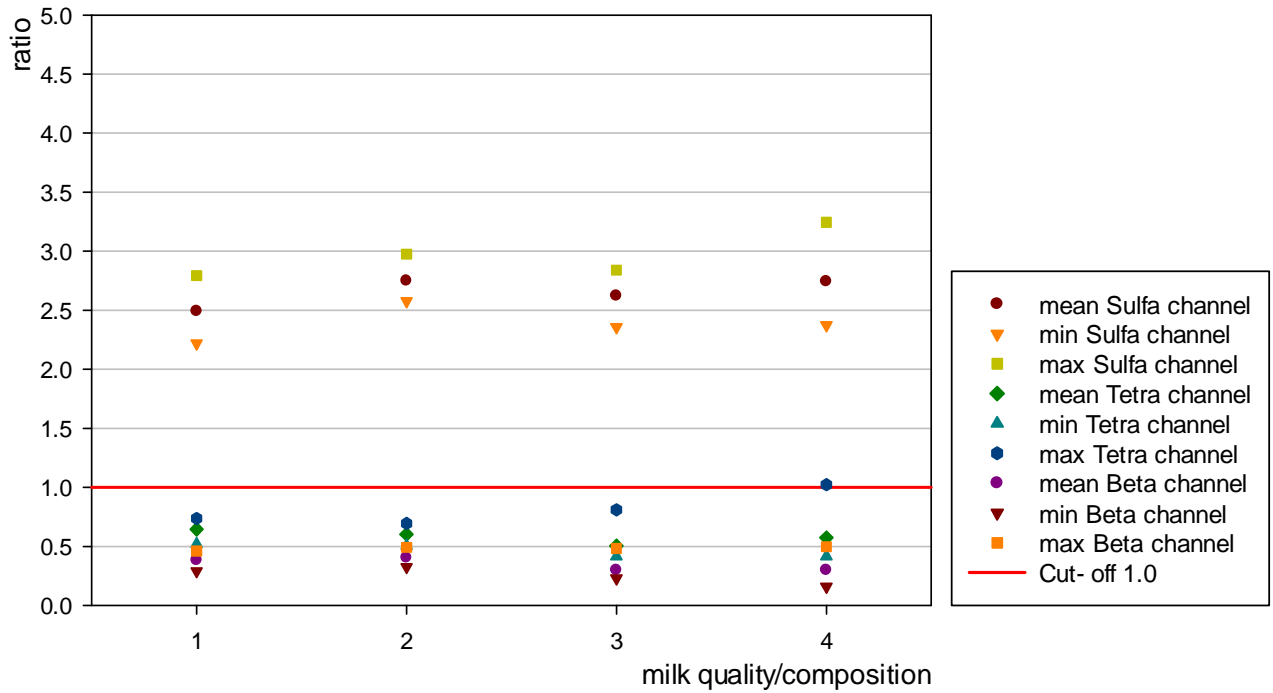


Fig. 5. Results for milk spiked with benzylpenicillin at 1.5 $\mu\text{g}/\text{kg}$ and oxytetracycline at 7 $\mu\text{g}/\text{kg}$, 10 replicates. Sulfa: sulfonamides, Tetra: tetracycline; Beta: β -lactam.

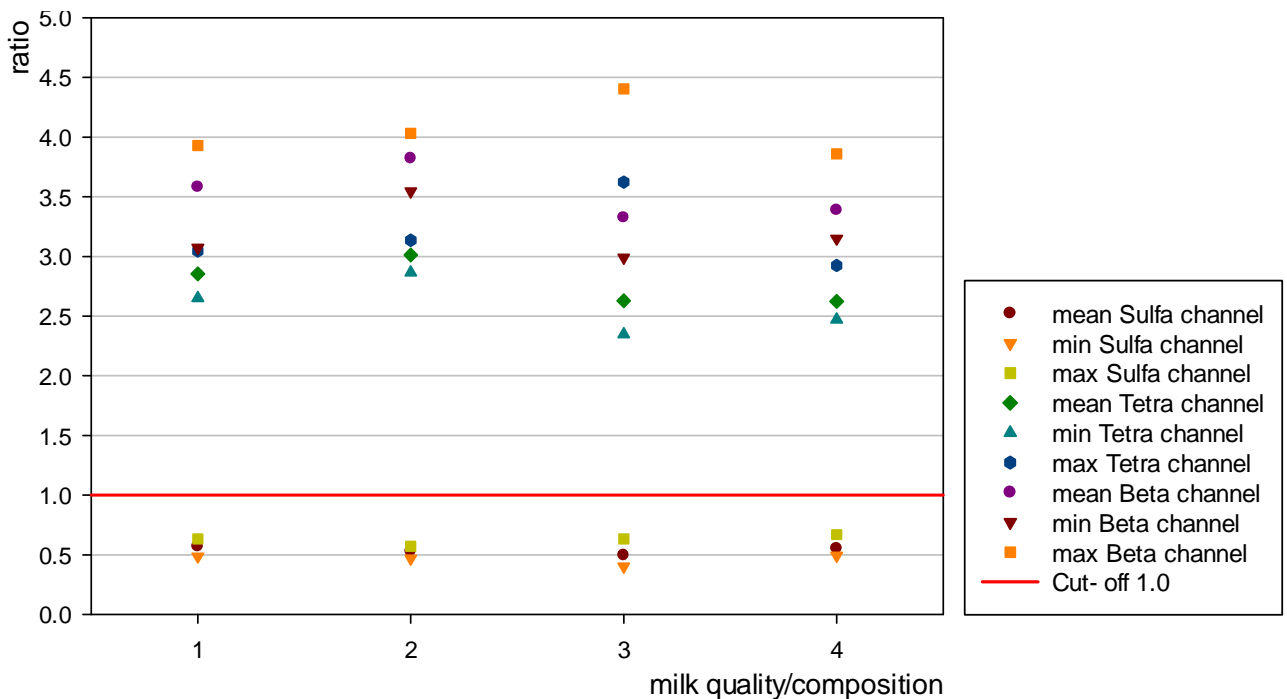


Fig. 6. Results for milk spiked with sulfadoxine at 3 $\mu\text{g}/\text{kg}$, 10 replicates. Sulfa: sulfonamides, Tetra: tetracycline; Beta: β -lactam.

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

	Ratio								
	Sulfa			Tetra			Beta		
	mean	min	max	mean	min	max	mean	min	max
Blank raw cows' milk									
Normal milk = reference	2.7660	2.3483	2.9508	3.1785	2.9238	3.3964	3.9156	3.1923	4.2087
High total bacterial count	2.9917	2.8080	3.1268	3.0774	1.3254	3.5617	4.2204	3.9221	4.6530
Low fat < 1.40g/100g	2.9428	2.6693	3.0703	3.0036	2.7217	3.8839	3.7516	3.3789	4.8620
Low protein < 2.99g/100ml	2.9473	2.6525	3.5101	2.8746	2.6011	3.6305	3.5876	3.2466	3.7657
Milk with benzylpenicillin at 1.5 µg/kg and oxytetracycline at 7 µg/kg									
Normal milk = reference	2.4921	2.2203	2.7948	0.6452	0.5193	0.7370	0.3818	0.2912	0.4605
High total bacterial count	2.7498	2.5783	2.9769	0.6028	0.5048	0.6955	0.4017	0.3260	0.4921
Low fat < 1.40g/100g	2.6220	2.3588	2.8410	0.5054	0.4136	0.8094	0.2985	0.2288	0.4816
Low protein < 2.99g/100ml	2.7433	2.3757	3.2461	0.5735	0.4148	1.0220	0.2994	0.1599	0.4972
Milk with sulfadoxine at 3 µg/kg									
Normal milk = reference	0.5695	0.4869	0.6335	2.8550	2.6509	3.0466	3.5817	3.0755	3.9296
High total bacterial count	0.5285	0.4679	0.5719	3.0130	2.8654	3.1367	3.8225	3.5460	4.0316
Low fat < 1.40g/100g	0.4947	0.4020	0.6331	2.6287	2.3494	3.6237	3.3251	2.9900	4.4046
Low protein < 2.99g/100ml	0.5524	0.4932	0.6699	2.6237	2.4728	2.9246	3.3881	3.1503	3.8611

Notes: min: minimum; max: maximum. Sulfa: sulfonamides, Tetra: tetracycline; Beta: β-lactam.

Discussion:

In general, a high total bacterial count or a low fat or protein content had mostly no significant influence on the performance of the AnticFast® Beta-lactams & Tetracyclines & Sulfonamides Rapid Test Kit result.

One blank milk sample with low protein content was found positive on the sulfonamide channel (ratio: 0.5879). The sample also tested positive on another screening test and LC-MS analysis (semi-quantitative) showed the presence of sulfadoxine at 3.0 µg/l and trimethoprim at 0.44 µg/l (result not included in Table 13 or Figure 4). So no false positives were obtained with the blank milk.

All positive samples were positive except for one milk sample with a low protein content spiked with benzylpenicillin at 1.5 µg/kg and oxytetracycline at 7 µg/kg, with one borderline negative result (ratio 1.0220) on the tetracycline channel. This indicates a small hampering of detection for low 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 and reconstituted milk powder were analysed in order to determine if the AnticFast® Beta-lactams & Tetracyclines & Sulfonamides Rapid Test Kit is a suitable test for these types of milk.

Results:

With respect to the impact of the milk type (UHT and reconstituted milk powder), 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 = Reconstituted milk powder.

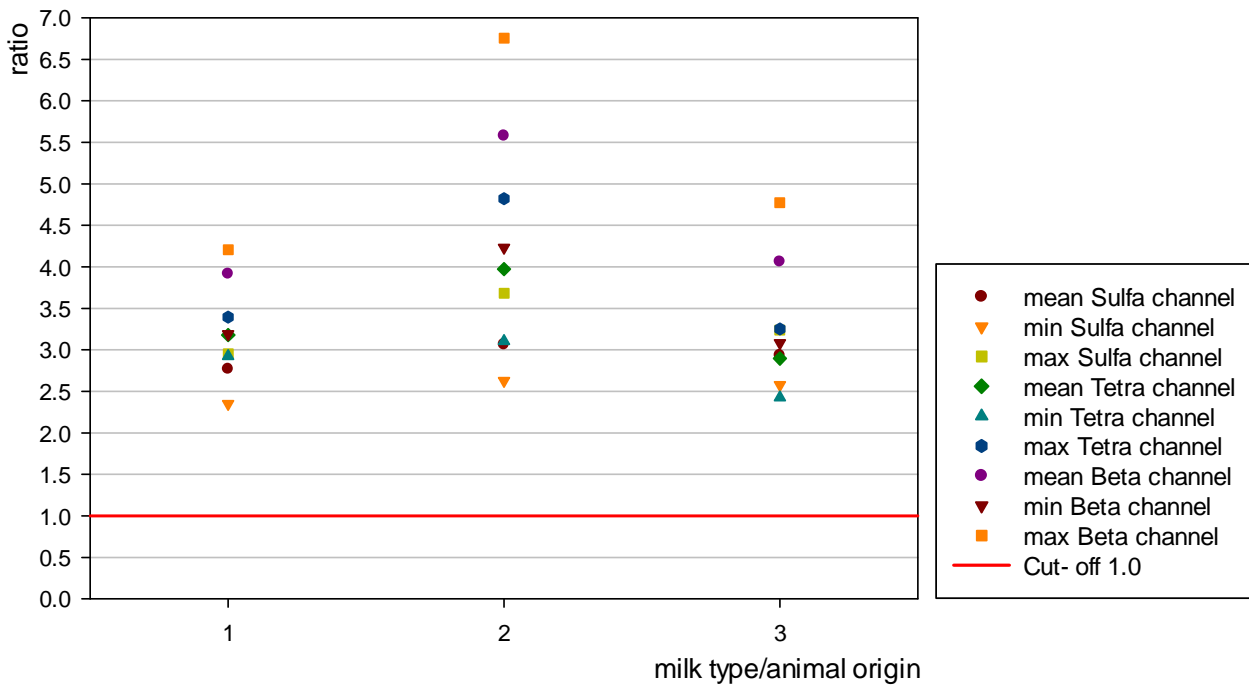


Fig. 7. Results for blank milk, 10 replicates. Sulfa: sulfonamides, Tetra: tetracycline; Beta: β -lactam.

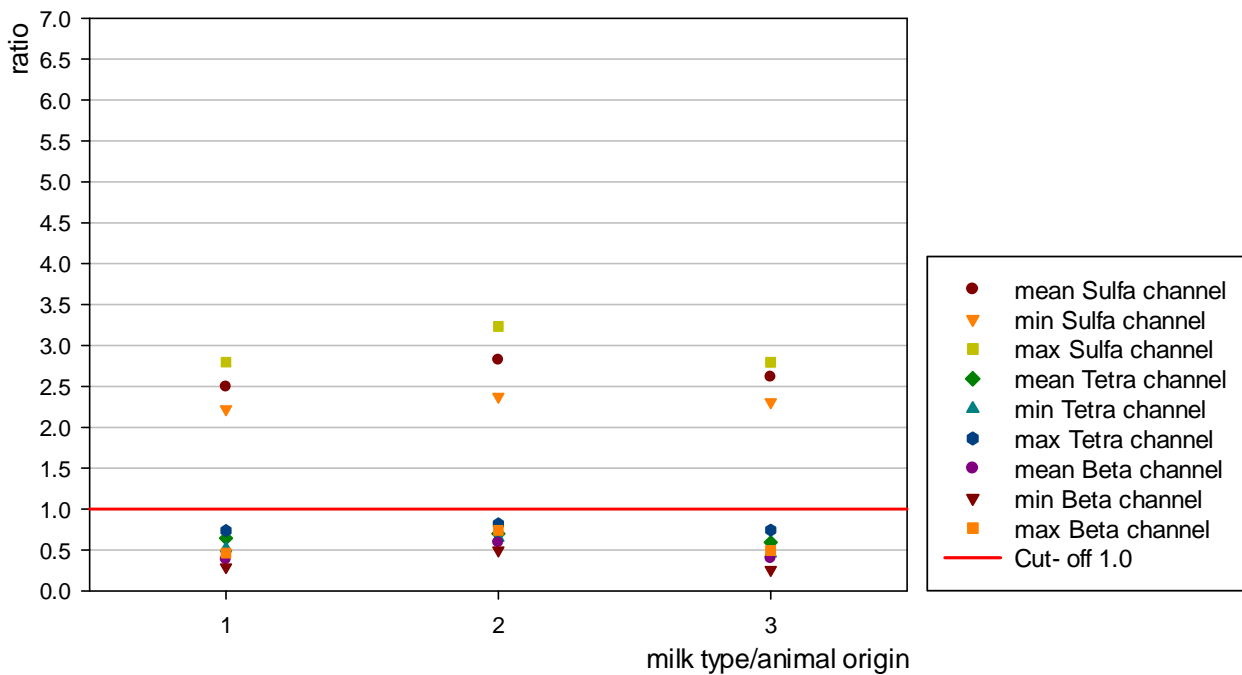


Fig. 8. Results for milk spiked with benzylpenicillin at 1.5 µg/kg and oxytetracycline at 7 µg/kg, 10 replicates. Sulfa: sulfonamides, Tetra: tetracycline; Beta: β-lactam.

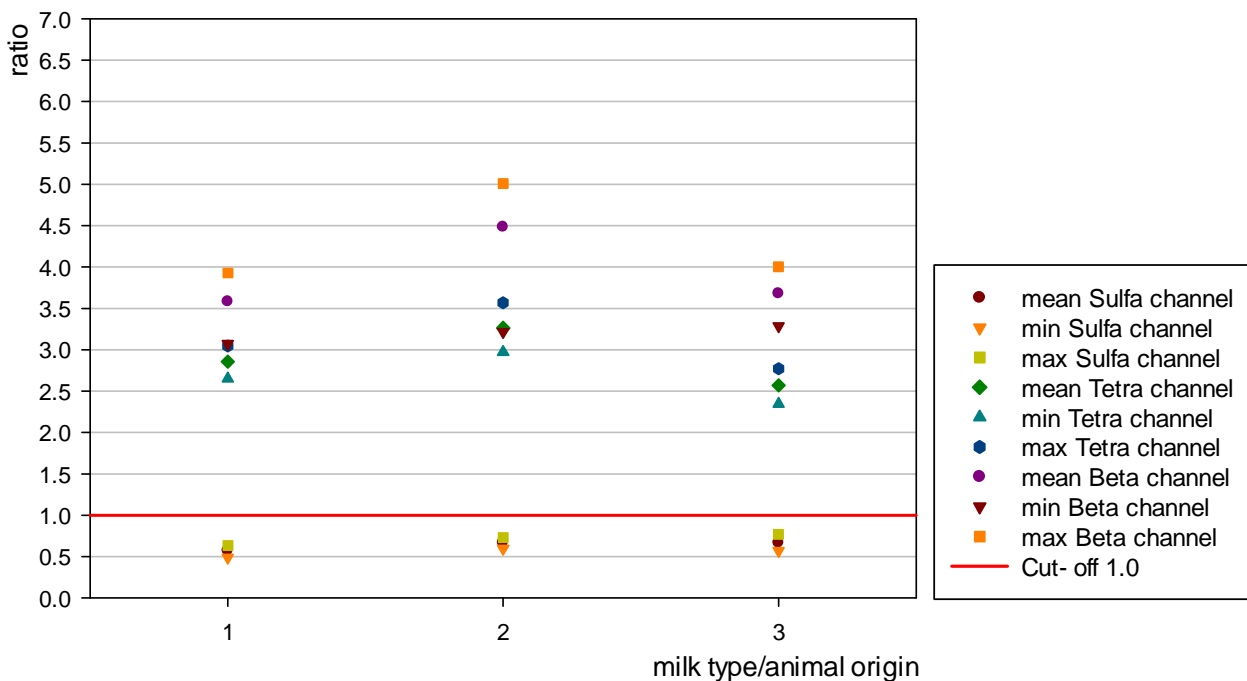


Fig. 9. Results for milk spiked with sulfadoxine at 3 µg/kg, 10 replicates. Sulfa: sulfonamides, Tetra: tetracycline; Beta: β-lactam.

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

Milk type	Ratio								
	Sulfonamides channel			Tetracycline channel			Beta-lactam channel		
	mean	min	max	mean	min	max	mean	min	max
Blank milk									
Normal raw cows' milk = reference	2.7660	2.3483	2.9508	3.1785	2.9238	3.3964	3.9156	3.1923	4.2087
UHT*	3.0647	2.6288	3.6827	3.9749	3.1072	4.8233	5.5787	4.2333	6.7549
Milk powde	2.9302	2.5762	3.2401	2.8941	2.4241	3.2522	4.0604	3.0840	4.7724
Milk with benzylpenicillin at 1.5 µg/kg + oxytetracycline at 7 µg/kg									
Normal raw cows' milk = reference	2.4921	2.2203	2.7948	0.6452	0.5193	0.7370	0.3818	0.2912	0.4605
UHT*	2.8216	2.3729	3.2349	0.6998	0.6441	0.8182	0.5884	0.4978	0.7396
Milk powder	2.6132	2.3067	2.7933	0.5926	0.4577	0.7422	0.3950	0.2579	0.4933
Milk with sulfadoxine at 3 µg/kg									
Normal raw cows' milk = reference	0.5695	0.4869	0.6335	2.8550	2.6509	3.0466	3.5817	3.0755	3.9296
UHT*	0.6643	0.5964	0.7307	3.2652	2.9688	3.5681	4.4829	3.8172	5.0081
Milk powder	0.6649	0.5720	0.7666	2.5698	2.3452	2.7719	3.6783	3.2883	4.0056

Notes: *: for UHT milk lighter control lines were obtained; min: minimum; max: maximum.

Discussion:

There could also be interest to use the AnticFast® Beta-lactams & Tetracyclines & Sulfonamides Rapid Test Kit, although developed for the testing of raw cows' milk, to test UHT milk or reconstituted milk powder.

For blank milk, all types of milk gave negative results. For spiked milk types always positive results were obtained. It was however noticed that for UHT milk, the control line was much lighter than usual.

The AnticFast® Beta-lactams & Tetracyclines & Sulfonamides Rapid Test Kit can be used to analyse UHT milk and reconstituted milk powder.

6.5. Stability of reagents – daily control samples

Methods and material:

The following control samples were analyzed daily with the AnticFast® Beta-lactams & Tetracyclines & Sulfonamides Rapid Test Kit to check the stability of the reagents and consistency of results:

- Blank milk (antibiotic-free raw milk)
- Raw milk spiked with 1.5 µg/kg of benzylpenicillin and oxytetracycline at 7 µg/kg
- Raw milk spiked with 3 µg/kg of sulfadoxine

Each day, also a negative and positive control (lot numbers and expiry dates are equal to those of the kit reagents (lot 1 20200918G (expiration date 18/09/2021) and lot 2 20200821G (expiration date 21/08/2021)) 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, 25 µg/kg of oxytetracycline and 25 µg/kg of sulfamethazine.

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.

Discussion:

Very stable ratio values were obtained for daily control samples with the AnticFast® Beta-lactams & Tetracyclines & Sulfonamides 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, except for one borderline negative result (ratio 1.0322) on the tetracycline channel.

For the last 4 results for blank milk and for both spiked milk samples, the improved reagents (lot 3: 20210909G (expiration date 09/09/2022) and lot 4: 20211009G (expiration date 09/10/22)) were used. For both blank as well as spiked samples, the ratios are lower, but always correct results were obtained.

The negative and positive controls inserted in the kit always gave correct results.

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

Standard	Ratio											
	Sulfonamide channel				Tetracycline channel				Beta-lactam channel			
	mean	min	max	S _r	mean	min	max	S _r	mean	min	max	S _r
Blank milk	2.7780	1.4669	3.2827	0.38	3.1776	1.6792	3.8363	0.49	4.0658	1.5939	4.8806	0.77
Milk spiked with benzylpenicillin at 1.5 µg/kg and oxytetracycline at 7 µg/kg	2.4596	1.2570	3.1103	0.42	0.6616	0.3755	1.0233	0.17	0.3798	0.2195	0.6563	0.11
Milk spiked with sulfadoxine at 3 µg/kg	0.5090	0.2962	0.7480	0.09	2.7817	1.4958	3.8855	0.50	3.5802	1.4094	5.2515	0.81
Controls included in the test kit												
Negative	2.5476	2.2027	2.7337	0.11	2.2450	1.6142	2.5773	0.25	2.7569	2.2092	3.0583	0.22
Positive	0.3159	0.2273	0.3847	0.04	0.1701	0.1182	0.2271	0.02	0.1267	0.0797	0.1775	0.02

Notes: S_r: standard deviation; min: lowest ratio; max: highest ratio.

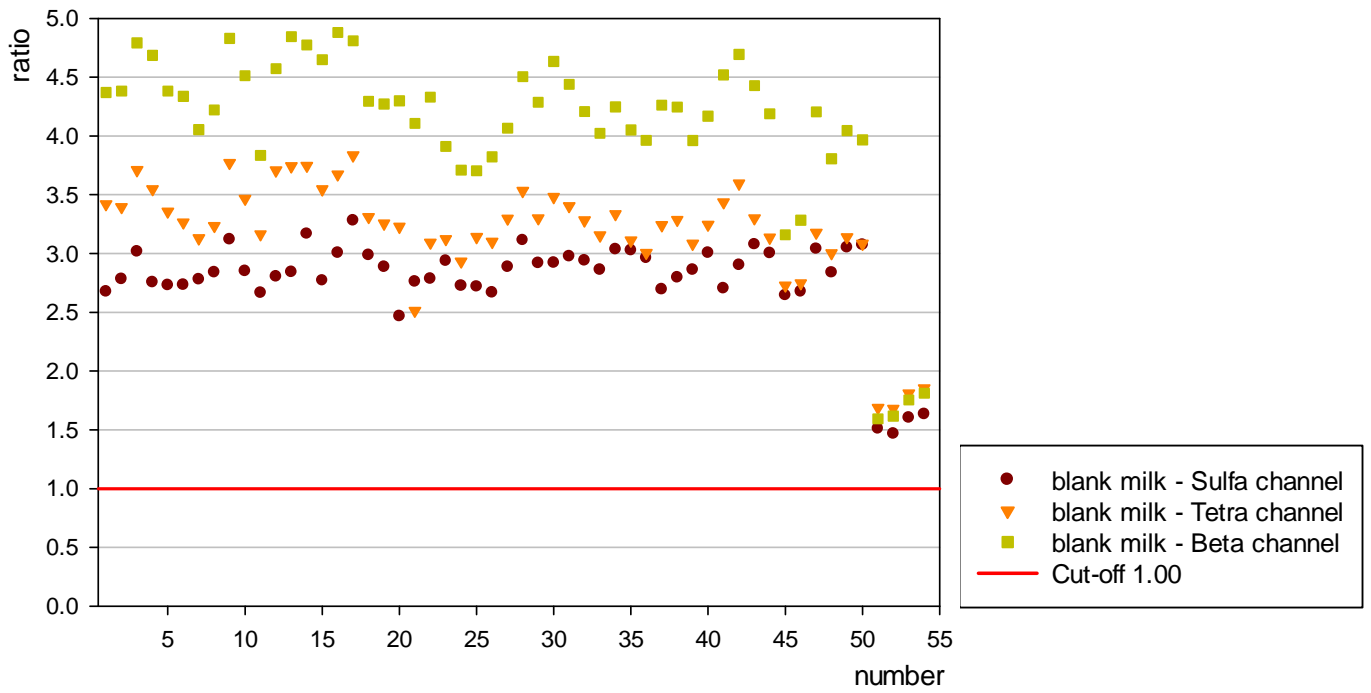


Fig. 10. AnticFast® Beta-lactams & Tetracyclines & Sulfonamides Rapid Test Kit results (ratio) for the blank control samples. Sulfa: sulfonamides, Tetra: tetracycline; Beta: β -lactam.

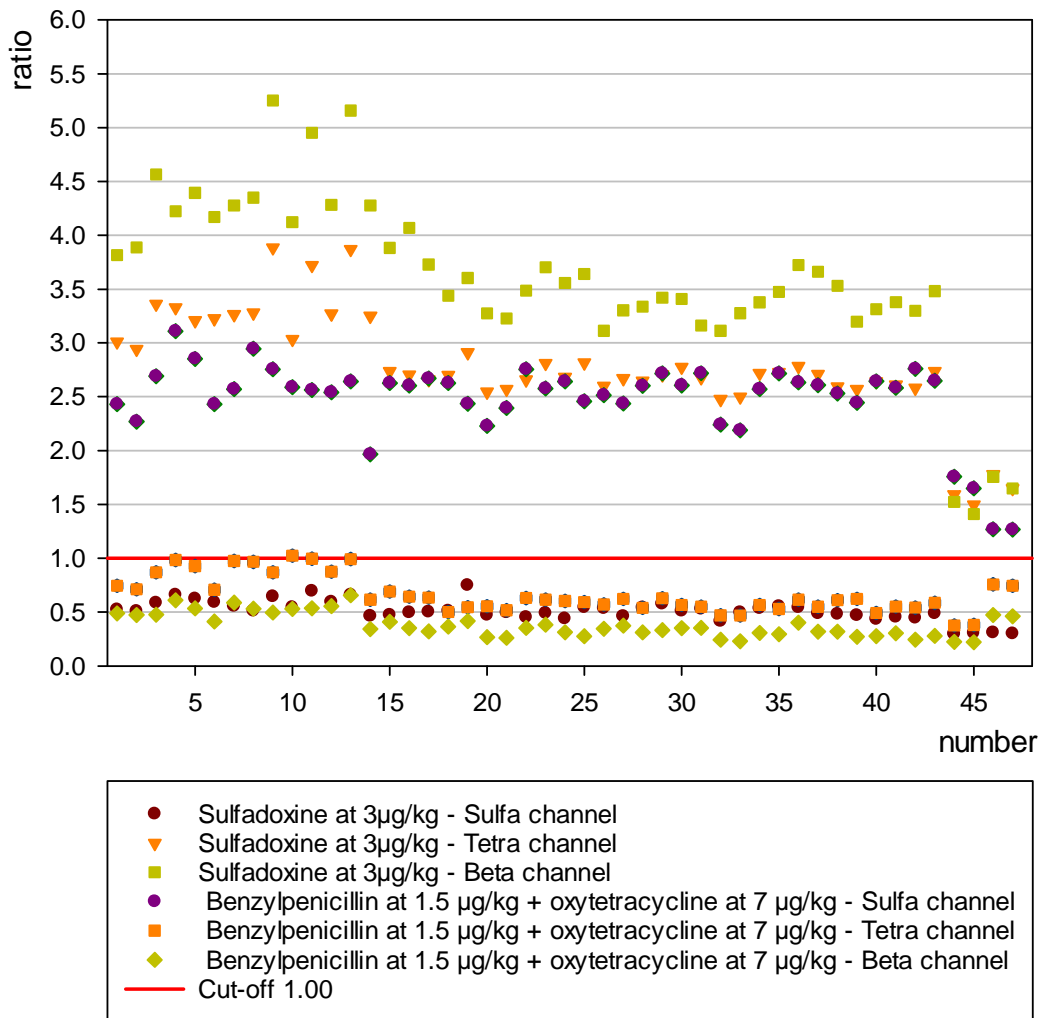


Fig. 11. AnticFast® Beta-lactams & Tetracyclines & Sulfonamides Rapid Test Kit results (ratio) for the spiked control samples with benzylpenicillin at 1.5 µg/kg and oxytetracycline at 7 µg/kg and spiked with sulfadoxine at 3 µg/kg. Sulfa: sulfonamides, Tetra: tetracycline; Beta: β-lactam.

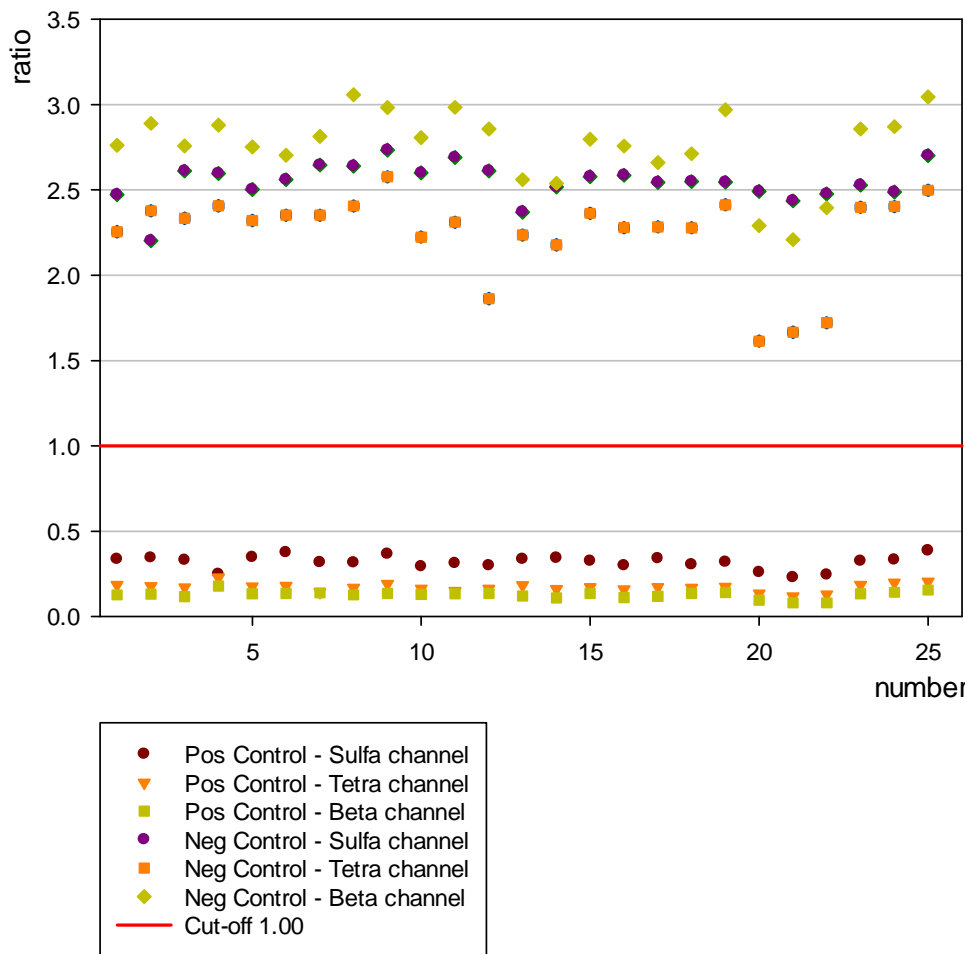


Fig. 12. AnticFast® Beta-lactams & Tetracyclines & Sulfonamides Rapid Test Kit results (ratio) for the negative and positive controls inserted in the kit. Sulfa: sulfonamides, Tetra: tetracycline; Beta: β -lactam.

7. Reliability of the instrumentation

In general no main problems were encountered with the instrumentation during validation. Points 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. For lot 20200918G, an interrupted beta-lactam test line was observed a few times. So it is important to check whether all test lines are well formed before interpreting the result.

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 & Sulfonamides Rapid Test Kit was integrated as rapid test.

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 16. Results of AnticFast® Beta-lactams & Tetracyclines & Sulfonamides Rapid Test Kit in the national ring trial of 22 April 2021 (Ooghe & Reybroeck, 2021).

LAB	Visual reading AnticFast® Beta-lactams & Tetracyclines & Sulfonamides Rapid Test Kit								Instrumental reading (ratio + result) AnticFast® Beta-Lactams & Tetracyclines & Sulfonamides 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			
SULFONAMIDES																			20200918G 18/09/2021 BMZ6000 Portable Strip Reader
ILVO	+	-	-	-	-	-	-	-	0.4808 POS	2.2678 NEG	2.1338 NEG	1.8771 NEG	2.2962 NEG	2.5012 NEG	2.5244 NEG	2.0956 NEG			
TETRACYCLINES																			
ILVO	-	-	-	-	+	-	-	-	3.0609 NEG	2.7839 NEG	2.4393 NEG	2.2886 NEG	0.3089 POS	3.0040 NEG	3.1547 NEG	2.3743 NEG			
BETA-LACTAM																			
ILVO	-	+	+	+	-	-	-	+	3.6586 NEG	0.4700 POS	0.2677 POS	0.2928 POS	2.9411 NEG	3.5815 NEG	3.7552 NEG	0.3650 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 & Sulfonamides Rapid Test Kit.

Except for sample F, spiked with cefalexin at 100 µg/kg, all other milk samples fortified with β-lactam antibiotics (samples B, C, D & H) were screened positive with AnticFast® Beta-lactams

& Tetracyclines & Sulfonamides Rapid Test Kit. This is in line with the detection capability reported by Meizheng Group for cefalexin (> MRL).

The milk sample spiked with chlortetracycline at 100 µg/kg (sample E) was screened positive on the tetracycline test line of AnticFast® Beta-lactams & Tetracyclines & Sulfonamides Rapid Test Kit and the milk sample fortified with sulfadoxine at 100 µg/kg (sample A) was screened positive on the sulfonamide test line of AnticFast® Beta-lactams & Tetracyclines & Sulfonamides Rapid Test Kit.

Hence, in this ring test sulfadoxine, cefquinome, cloxacillin, benzylpenicillin, chlortetracycline and ampicillin are detected at MRL with AnticFast® Beta-lactams & Tetracyclines & Sulfonamides 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 & Sulfonamides Rapid Test Kit.

9. Final conclusion

Results of this validation show that the AnticFast® Beta-lactams & Tetracyclines & Sulfonamides 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 cephalosporins), tetracyclines and sulfonamides. With the improved reagents (lot 20210909G (expiration date 09/09/2022) and lot 20211009G (expiration date 09/10/22)), all β-lactams can be detected at least in 95% of the replicates at their respective MRL except for cefalexin and desfuoylceftiofur. The exact detection capability (above MRL) of cefalexin and desfuoylceftiofur was not determined. All tetracyclines, including 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. All tested sulfonamides are detected at least in 95% of the replicated at their respective MRL.

Next to raw cows' milk, AnticFast® Beta-lactams & Tetracyclines & Sulfonamides Rapid Test Kit is suitable to be used to screen UHT milk and reconstituted milk powder.

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REFERENCES

Anonymous. 2005. Martindale: The complete drug reference. Ed. Sweetman S. C. Royal Pharmaceutical Society of Great Britain, Pharmaceutical Press, London, United Kingdom, 34th Edition.

Anonymous. 2007. CRLs view on state of the art analytical methods for national residue control plans. CRL Guidance Paper (December 7, 2007): 1-8.

Anonymous. 2010. Guidelines for the validation of screening methods for residues of veterinary medicines (initial validation and transfer). Community Reference Laboratories Residues (CRLs). 20/01/2010: 1-18.

Anonymous. 2020. Kit insert AnticFast® Beta-lactams & Tetracyclines & Sulfonamides Rapid Test Kit. Ver. 2020-1.

Anonymous. 2021. Tests rapides et tests inhibiteurs microbiologiques à utiliser pour la détection des substances inhibitrices dans le lait cru dans le cadre de l'autocontrôle. 15/12/2021. <http://www.afsca.be/productionanimale/produitsanimaux/circulaires/>

Commission Decision (EC) No 2002/657 of 12 August 2002 implementing Council Directive 96/23/EC concerning the performance of analytical methods and the interpretation of results. Off. J. Eur. Comm. 2002 L221: 8-36.

Commission Decision (EC) No 2003/181/EC of 13 March 2003 as regards the setting of minimum required performance limits (MRPLs) for certain residues in food of animal origin. Off. J. Eur. Union 2003 L71: 17-18.

Commission Regulation (EU) No 37/2010 of 22 December 2009 on pharmacologically active substances and their classification regarding maximum residue limits in foodstuffs of animal origin. Off. J. Eur. Union 2010 L15: 1-72.

ISO/IDF. 2020. Ballot document - ISO/DTS 23758 | IDF/RM 251 - Guidelines for the validation of qualitative screening methods for the detection of residues of veterinary drugs in milk and milk products. 2020-09-01: 1-43.

Ooghe S., Reybroeck W. 2021. Rapport ringonderzoek antibiotica screening in melk: snelsten en microbiologische inhibitortesten: April 22, 2021. ILVO-T&V Melle. Belgium:1-49.

Regulation (EC) No 470/2009 of the European Parliament and of the Council of 6 May 2009 laying down Community procedures for the establishment of residue limits of pharmacologically active substances in foodstuffs of animal origin, repealing Council Regulation (EEC) No 2377/90 and amending Directive 2001/82/EC of the European Parliament and of the Council and Regulation (EC) No 726/2004 of the European Parliament and of the Council laying down a Community procedure for the establishment of maximum residue limits of veterinary medicinal products in foodstuffs of animal origin. Off. J. Eur. Union 2009 L152: 11-22.

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