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Validation report AnticFast® Beta- lactams Rapid Test Kit

(Order n°: JC0056)

(Meizheng Bio-Tech, China)

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1. Introduction

AnticFast® Beta-lactams Rapid Test Kit (Meizheng Bio-Tech, China) is a qualitative two-step (2 min + 5 min) rapid lateral flow assay to detect β -lactam (penicillins and cephalosporins) 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 will also be included in next inter-laboratory study organized by ILVO in 2021.

Update of the report: determination of the detection capacity for ceftiofur with improved reagents (lot 3: 20210920G (expiration date 20/09/2022) and lot 4: 20211012G (expiration date 12/10/22)). All other validation (initial report) was performed using reagents lot 1: 20200815G (expiration date 15/08/2021) and lot 2: 20200901G (expiration date 01/09/2021).

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). Raw milk temperature should be 2-15°C. In this validation study, standard raw milk of 2-4°C is used.

Remove the cover of the tube and take out the appropriate number of test strips and microwells. Immediately cover the tube and restore the remaining components at 2-8°C.

Test procedure

1st step: Turn on the HMG-GS Microwell Incubator and wait until the incubator is stable at 40°C. Place the empty microwells into the incubator.

2nd step: 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.

3rd step: Incubate the sample for 2 minutes at 40°C, then insert the test strip into the microwell.

4th step: Let the test strip develop color for 5 minutes at 40°C.

5th step: Take out the strip and remove the absorbent pad. Interpret the results within 1 minute visually or by using a BMZ6000 Portable Strip Reader and software.

For the test line (T1) following counts: Negative: two lines are visible. 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 Rapid Test Kit test strip

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

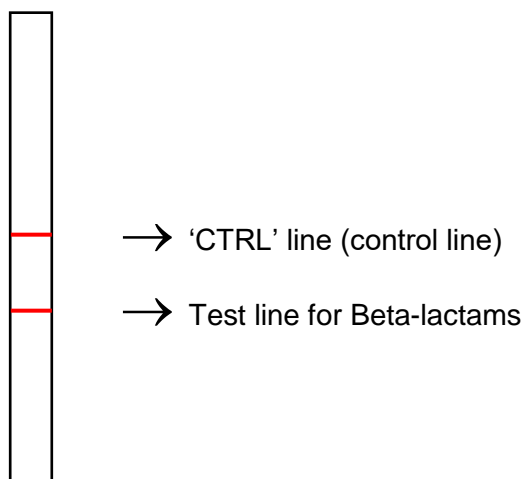


Fig. 1. Configuration of an AnticFast® Beta-lactams Rapid Test Kit test strip.

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, a 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
$R \geq 1.00$	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 Rapid Test Kit.

2.3 Visual interpretation of the test

The kit manufacturer claims that visual reading of the AnticFast® Beta-lactams Rapid Test Kit is possible. The intensity of the test line is compared to the intensity of the reference (i.e. control) line. Negative: two lines are visible. 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.

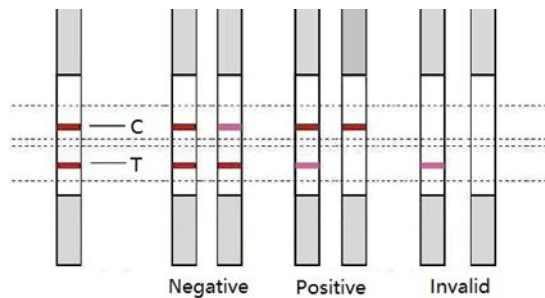


Fig. 3. Visual interpretation of the color formation on the AnticFast® Beta-lactams Rapid Test Kit (Anon.; 2019).

3. Detection capability

Methods and Materials:

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

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 Rapid Test Kit was determined for all different compounds belonging to the β -lactam 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 $> \text{MRL}$: 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 $\mu\text{g}/\text{kg}$ - twice

- blank raw milk spiked with cefalonium at 1 µg/kg - twice

Detection capability tests were performed with 4 different lots of reagents (lot 1 20200815G (expiration date 15/08/2021) and lot 2 20200901G (expiration date 01/09/2021), and for the detection capability of ceftiofur, two additional (more sensitive) lots were used: lot 3 20210920G (expiration date 20/09/2022) and lot 4 20211012G (expiration date 12/10/22). All were used following the manufacturer's instructions. The intensity of color formation of each test line was compared to the intensity of the control line and was interpreted by means of a BMZ6000 Portable Strip Reader and software. The cut-off value is 1.00 (≥ 1.00 : negative; < 1.00 : positive). All results (reader values) were collected in a data base.

Table 3. Standard material used in this validations study.

Compound	Origin	Product number	Lot number
Amoxicillin trihydrate	Sigma-Aldrich	31586	BCCB1309
Ampicillin trihydrate	Sigma-Aldrich	31591	BCBS3642V
Cefacetile	Sigma-Aldrich	C231500	2-MAX-147-3
Cefalexin	Sigma-Aldrich	33989-100MG-R	lot BCBW7031
Cefalonium hydrate	Sigma-Aldrich	32904	BCBV1595
Cefapirin sodium	Sigma-Aldrich	43989	BCCC5208
Cefapirin sodium	Sigma-Aldrich	43989	BCBV1345
Cefazolin European Pharmacopeia reference standard	Sigma-Aldrich	C0682800	5.0
Cefoperazone dihydrate	Sigma-Aldrich	32426	BCBX0019
Cefquinome sulfate	Sigma-Aldrich	32472	BCBW2550
Ceftiofur	Dr Ehrenstorfer	DRE-C11065000	G1104213
Chloramphenicol VETRANAL	Sigma-Aldrich	31667	BCBR6685V
Chlortetracycline hydrochloride	Sigma-Aldrich	46133	BCBT9837
Clavulanic acid (Potassium clavulante)	Sigma-Aldrich	33454	STBJ0056
Cloxacillin sodium salt monohydrate	Sigma-Aldrich	C9393	016M4853V
Colistin sulfate	Sigma-Aldrich	C4461	049M4836V
Dapsone	Sigma-Aldrich	46158	BCBX0187
Desacetylcephapirin	LGC standards	682120	799058
Desfuoylceftiofur	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
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
Penicillin G sodium salt	Sigma-Aldrich	PENNA	045M4815V
Sulfadiazine	Sigma-Aldrich	S8626	056M4795V
Tetracycline hydrochloride	Sigma-Aldrich	31741	BCCC9767
Trimethoprim, minimum 98%TLC	Sigma-Aldrich	46984	BCBX8831

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

Results:

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

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

Group	Marker residue	MRL (µg/kg)	Detection capability	
			Lot 1 and 2 (µg/kg)	Lot 3 and 4 (µg/kg)
Penicillins	Benzylpenicillin	4	1	
	Ampicillin	4	3	
	Amoxicillin	4	3	
	Oxacillin	30	4	
	Cloxacillin	30	3	
	Dicloxacillin	30	3	
	Nafcillin	30	14	
Cephalosporins	Ceftiofur	100 ^a		80
	Desfuroylceftiofur	100 ^a	-- (>MRL) *	
	Cefquinome	20	12	
	Cefazolin	50	45	
	Cephapirin	60 ^b	12	
	Desacetylcephapiri n	60 ^b	30	
	Cefacetile	125	30	
	Cefoperazone	50	2	
	Cefalexin	100	-- (>MRL) *	
	Cefalonium	20	1	

Notes: lot 1: 20200815G (expiration date 15/08/2021); lot 2: 20200901G (expiration date 01/09/2021), lot 3: 20210920G (expiration date 20/09/2022) and lot 4: 20211012G (expiration date 12/10/22).

*: 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 desfuoylceftiofur,

^b: The MRL of 60 µg/kg in milk is applied on the sum of cephalosporins and desacetylcephalosporins.

Discussion:

All β-lactams (penicillins and cephalosporins) present on the EU-MRL list in milk (Commission Regulation (EU) No 37/2010) as included in Table 4 can be detected at their respective MRL by AnticFast® Beta-lactams Rapid Test Kit (improved reagents) at least in 95% of the replicates except for desfuoylceftiofur and cefalexin. The 95% detection capability for these two compounds was not determined as these compounds could not be detected at MRL and since not requested by the kit manufacturer.

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 CC_β of 80 µg/kg was determined for ceftiofur. The impact of the improvement of the reagents on the CC_β for the other β-lactams was not determined. 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 Rapid Test Kit was tested by analysing milk doped with analysing compounds belonging to different antibiotic or chemotherapeutic families (1 per family) to check the selectivity of the β-lactam test line. Raw milk was doped 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: 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).

All standard material from Sigma-Aldrich was used (Table 3).

Results:

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

Discussion:

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

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

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

Family	Compound	MR(P)L ($\mu\text{g}/\text{kg}$)	Conc. spiked in milk ($\mu\text{g}/\text{kg}$)	Ratio	Result
Tetracyclines	Oxytetracycline	100 ^a	10,000	1.8001	-
Sulfonamides	Sulfadiazine	100 ^b	10,000	1.9372	-
Aminoglycosides	Neomycin B	1,500	150,000	1.7353	-
Macrolides	Erythromycin	40	4,000	1.9985	-
Quinolones	Enrofloxacin	100 ^c	10,000	1.8526	-
Amphenicols	Chloramphenicol	0.3 ^d	30	1.8872	-
Polymyxins	Colistin	50	5,000	1.9916	-
Lincosamides	Lincomycin	150	15,000	1.8332	-
β -lactamase inhibitors	Clavulanic acid	200	20,000	0.1459	+
Diamino pyrimidine derivatives	Trimethoprim	50	5,000	1.9573	-
Others	Dapsone	5 ^e	500	1.9670	-

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.

^a: The MRL of 100 $\mu\text{g}/\text{kg}$ 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 $\mu\text{g}/\text{kg}$

^c: The MRL of 100 $\mu\text{g}/\text{kg}$ 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 blank farm and 302 tanker load milk samples were tested with AnticFast® Beta-lactams Rapid Test Kit and other microbiological and receptor screening tests.

Results and discussion:

All 300 farm milk samples tested negative for β -lactams on AnticFast® Beta-lactams Rapid Test Kit. Of the 302 tanker load milk samples, two samples tested positive of which one was proven to be a real positive by other screening tests. Repetition of the other positive tested sample (ratio: 0.9194) gave another positive (ratio: 0.9424) and one negative (ratio: 1.0209) result on AnticFast® Beta-lactams Rapid Test Kit. Other screening tests gave negative results, but were not able to detect all Beta-lactams at such a low levels as the AnticFast® Beta-lactams Rapid Test Kit. The sample gave borderline results and with LC-MS analysis, a very small concentration of 0.55 $\mu\text{g}/\text{kg}$ of cefalonium was found (cc β cefalonium AnticFast® Beta-lactams Rapid Test Kit = 1 $\mu\text{g}/\text{kg}$). Taking into account these results, it is concluded that in total no false positive results were obtained upon 602 samples. The results are summarized in Table 6.

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

	Farm milk (n=300)	Tanker milk (n=300)*
	Ratio	Ratio
Mean	1.8599	1.9121
Min	1.4553	1.3702
Max	2.3976	2.1708
s_r	0.11	0.10
CV%	6.17	5.01

Notes: mean: mean ratio; min: lowest ratio; max: highest ratio; s_r ; Standard Deviation; CV(%): Relative Standard Deviation; *: two samples excluded since indication of presence of residues of β -lactams.

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 doped 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 Rapid Test Kit results are summarized in Table 7.

Table 7. Repeatability of the reader

Reader repeatability	Beta-lactam		
	Mean ratio	s _r	CV%
Blank milk	1.8461	0.03	1.70
Low positive milk	0.8515	0.01	0.69
High positive milk	0.4508	0.00	0.76

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

Discussion:

The repeatability of the reader was very good; very low standard deviations of repeatability were obtained (highest value 1.70%).

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 doped samples, any compound found positive could be used for the testing of channel.

Results:

The results of the repeatability of the AnticFast® Beta-lactams Rapid Test Kit are summarized in Table 8.

Table 8: Repeatability of the test

Test repeatability	Beta-lactam		
	Mean ratio	s _r	CV%
Blank milk	1.8457	0.05	2.54
Low positive milk	0.7575	0.02	2.61
High positive milk	0.2433	0.01	3.80

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

Discussion:

The repeatability of the test was also very good, very low standard deviation values were obtained. The highest variance value is 3.80%.

6. Test robustness

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

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

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

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

Methods and Materials:

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

Results:

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

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

Ratio	Incubation time								
	1 min 45 sec + 5 min 30 sec	1 min 45 sec + 5 min	1 min 45 sec + 4 min 30 sec	2 min + 4 min 30 sec	2 min + 5 min (REF)	2 min + 5 min 30 sec	2 min 15 sec + 4 min 30 sec	2 min 15 sec + 5 min	2 min 15 sec + 5 min 30 sec
Blank milk									
mean	1.8612	1.7623	1.8990	1.9368	1.8243	1.8360	1.8702	1.8415	1.8449
min	1.7583	1.6670	1.8064	1.9110	1.7337	1.8055	1.7961	1.8163	1.8083
max	1.9681	1.8108	1.9530	1.9776	1.8886	1.8539	1.9339	1.8716	1.8844
Milk doped with benzylpenicillin at 1 µg/kg									
mean	0.4191	0.4491	0.4477	0.3997	0.4886	0.4269	0.4053	0.3689	0.3864
min	0.4093	0.4274	0.4171	0.3684	0.4502	0.3929	0.3868	0.3383	0.3554
max	0.4245	0.4755	0.4744	0.4263	0.5168	0.4702	0.4312	0.3846	0.4028
Milk doped with cefalonium at 1 µg/kg									
mean	0.6468	0.6091	0.6531	0.6170	0.6706	0.6024	0.5510	0.5521	0.5622
min	0.5640	0.5557	0.5829	0.5730	0.6514	0.5482	0.5012	0.5294	0.5429
max	0.6885	0.6516	0.6987	0.6468	0.6845	0.6555	0.5870	0.5649	0.5744

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

Discussion:

Variations in the length of the incubation steps did not impact results significantly; all negative results remained negative and all positive results stayed positive. Doped milk did show slightly

lower ratios (become more positive) when the length of incubation is deviating from the standard 2' + 5'.

6.1.2. Delay of reading

Methods and Materials:

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

Results:

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

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

Ratio	Delay of reading		
	No delay (REF)	5 min	10 min
Blank milk			
mean	1.8327	1.8168	1.8080
min	1.8171	1.7839	1.7933
max	1.8475	1.8357	1.8291
Milk doped with benzylpenicillin at 1 µg/kg			
mean	0.3837	0.4089	0.4217
min	0.3539	0.3956	0.3608
max	0.3992	0.4352	0.4678
Milk doped with cefalonium at 1 µg/kg			
mean	0.5394	0.5551	0.5644
min	0.4648	0.4808	0.4660
max	0.6171	0.6406	0.6702

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

Discussion:

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

6.1.3. Volume of the milk

Methods and Materials:

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

Results:

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

Discussion:

A volume of milk differing some 20 µl (10%) from the prescribed volume of 200 µl did not impact the interpretation of test results; the negative results remained negative and positive results stayed positive. For the doped milk, a milk volume of 180 µl gave decreased ratios (more positive).

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

Ratio	Volume of milk		
	180 µl	200 µl (REF)	220 µl
Blank milk			
mean	1.9173	1.8327	1.9063
min	1.8477	1.8171	1.8729
max	2.0082	1.8475	1.9297
Milk doped with benzylpenicillin at 1 µg/kg			
mean	0.2465	0.3837	0.3609
min	0.1975	0.3539	0.3404
max	0.2815	0.3992	0.3899
Milk doped with cefalonium at 1 µg/kg			
mean	0.3320	0.5394	0.4674
min	0.2920	0.4648	0.4069
max	0.3760	0.6171	0.5127

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

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 Rapid Test Kit result.

Besides blank milk also doped milk samples containing benzylpenicillin at 1 µg/kg or doped milk containing cefalonium at 1 µg/kg were used.

Results:

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

Discussion:

The milk temperature (20°C) did not significantly impact the AnticFast® Beta-lactams Rapid Test Kit results: blank milk was always tested as negative while the doped milk samples gave a clear positive result on their respective channel.

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

Sample	Milk temperature	
	1-4°C (REF)	20°C
Blank milk		
mean	1.8327	1.8515
min	1.8171	1.7949
max	1.8475	1.8924
Milk doped with benzylpenicillin at 1 µg/kg		
mean	0.3837	0.3442
min	0.3539	0.2940
max	0.3992	0.3794
Milk doped with cefalonium at 1 µg/kg		
mean	0.5394	0.5300
min	0.4648	0.4336
max	0.6171	0.6197

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

6.3. Milk composition and milk type influences

Methods and Materials:

Fat content

Normal milk samples and milk samples with a low (<1.79 g per 100 ml) and high (>6.04 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 Rapid Test Kit result. The milk samples with a low and high fat content were natural milk samples with a low or high fat content 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

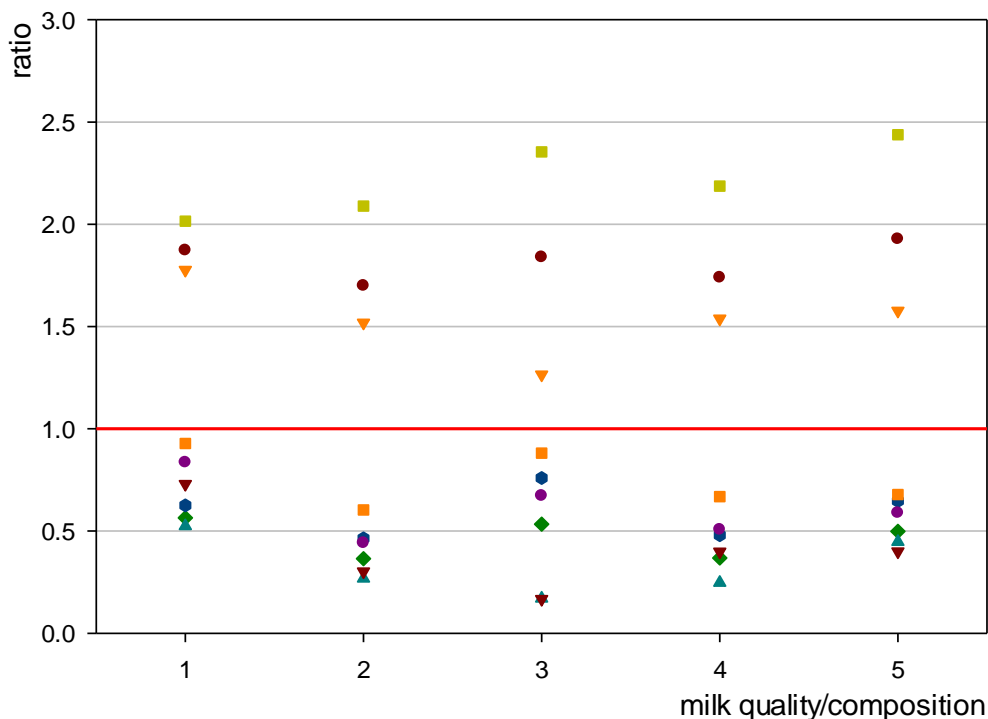
Normal milk samples and milk samples with a low (<2.98 g per 100 ml) or a high (>4.01 g per 100 g) protein content were analysed and the ratios obtained were compared in order to study the impact of the protein content on the AnticFast® Beta-lactams Rapid Test Kit result. The milk samples tested were 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 Figure 4 and Table 13.

The legend for the different situations in figure 4.

- 1 = Reference: normal raw milk;
- 2 = Low fat content (<1.79 g/100 ml);
- 3 = High fat content (>6.04 g/100 ml);
- 4 = Low protein (<2.98 g/100 ml);
- 5 = High protein (>4.01 g/100 ml);



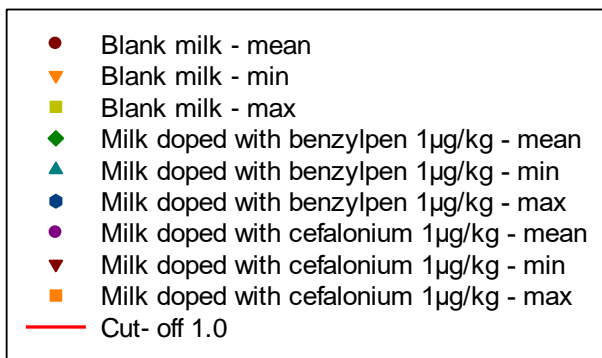


Fig. 4. Results for blank milk and milk doped with benzylpenicillin at 1 µg/kg and doped with cefalonium at 1 µg/kg, 10 replicates.

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

	Ratio		
	mean	min	max
Blank raw cows' milk			
normal milk = reference	1.8723	1.7769	2.0153
low fat <1.79 g/100 ml	1.6997	1.5196	2.0894
high fat >6.04 g/100 ml	1.8386	1.2649	2.3539
low protein <2.98 g/100 ml	1.7396	1.5391	2.1872
high protein >4.01 g/100 ml	1.9269	1.5777	2.4382
Milk with benzylpenicillin at 1 µg/kg			
normal milk = reference	0.5646	0.5241	0.6253
low fat <1.79 g/100 ml	0.3643	0.2675	0.4645
high fat >6.04 g/100 ml	0.5336	0.1699	0.7589
low protein <2.98 g/100 ml	0.3680	0.2470	0.4798
high protein >4.01 g/100 ml	0.4984	0.4472	0.6463
Milk with cefalonium at 1 µg/kg			
normal milk = reference	0.8341	0.7298	0.9282
low fat <1.79 g/100 ml	0.4411	0.3020	0.6030
high fat >6.04 g/100 ml	0.6713	0.1678	0.8808
low protein <2.98 g/100 ml	0.5059	0.3989	0.6681
high protein >4.01 g/100 ml	0.5872	0.4001	0.6786

Notes: min: minimum; max: maximum.

Discussion:

In general, the fat content and protein content had no significant influence on the performance of the AnticFast® Beta-lactams Rapid Test Kit result. No false positives were obtained with the blank milk. And all positive samples were positive. However, it was noticed that either a high or low fat or protein content resulted in a slightly larger range of ratios for both blank as well as doped milk. For milk doped at 1 µg/kg benzylpenicillin, lower ratios (more positive) were obtained for low fat and low protein milk. For milk doped at 1 µg/kg cefalonium, lower ratios were obtained for both low and high fat or protein milk.

6.4. Type of milk and animal origin influences

Methods and Materials:

Raw milk, UHT milk, sterilized milk and reconstituted milk powder were analysed in order to determine if the AnticFast® Beta-lactams 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 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 5 and 6 and Table 14.

The legend for the different situations in Figure 5 and 6:

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

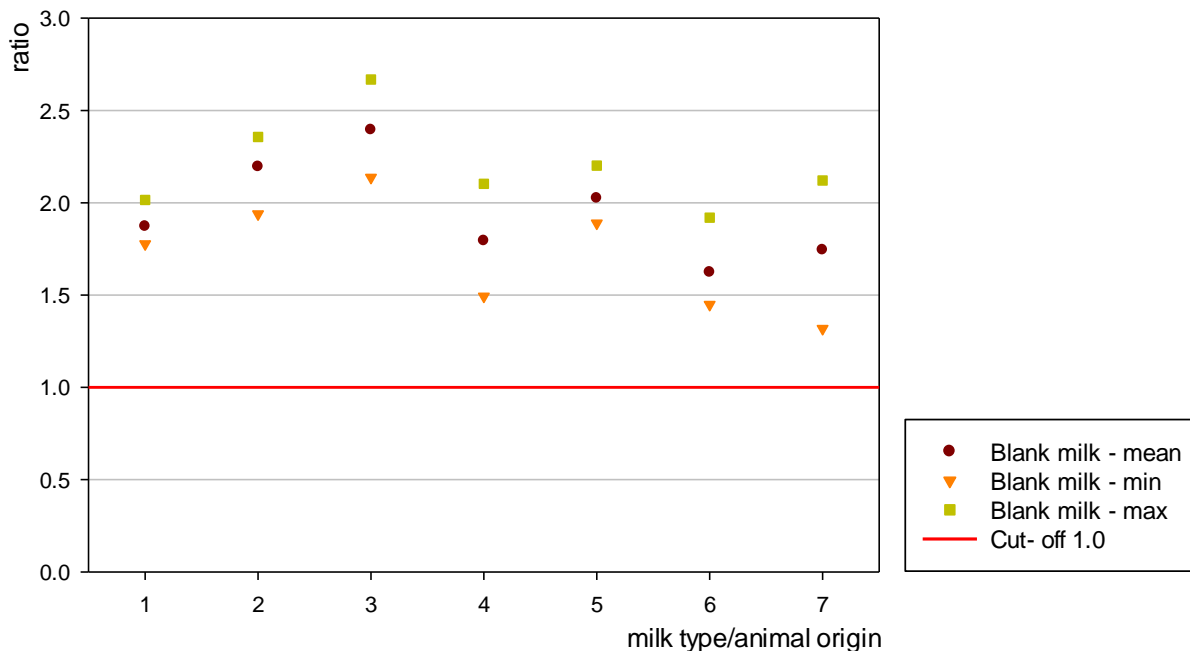


Fig. 5. Results for blank milk, 10 replicates.

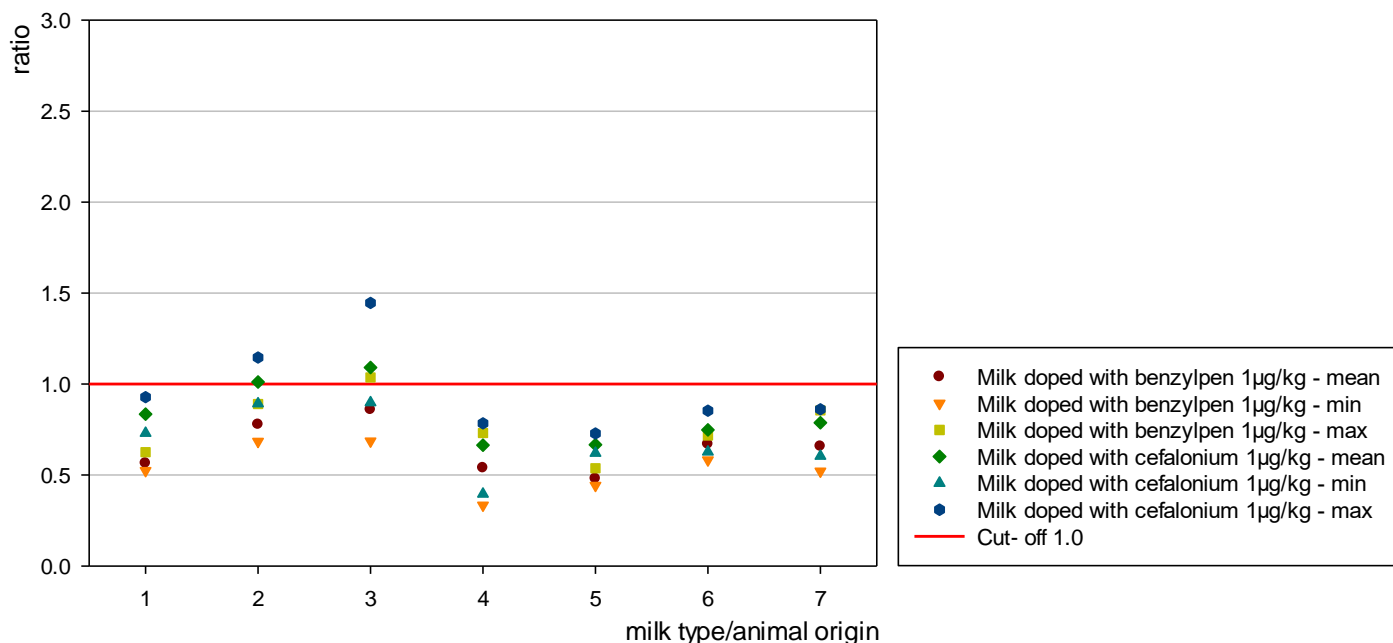


Fig. 6. Results for milk doped with benzylpenicillin at 1 µg/kg and milk doped with cefalonium at 1 µg/kg, 10 replicates.

Discussion:

There could also be interest to use the AnticFast® Beta-lactams 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 doped with benzylpenicillin at 1 µg/kg or with cefalonium at 1 µg/kg, UHT and sterilized milk samples obtained some negative results (5 and 9 negative results out of 20 doped samples, respectively). Therefore it can be concluded that an interference of detection occurs with heat treated milk such as UHT and sterilized milk. For UHT milk doped with 1 µg/kg benzylpenicillin, 2 borderline negative results were obtained. For UHT and sterilized milk doped with cefalonium, 5 and 7 negative results were obtained, respectively. For these compounds tested at concentrations far below MRL there are no problems to get detection at MRL. The same is valid for most other β-lactams with a CCβ far below MRL; just the detection capability of ampicillin and amoxicillin should be verified in heat-treated milk to confirm detection at MRL. For doped thawed milk and reconstituted milk samples, all positive results were obtained. However, for doped reconstituted milk powder, a diminished flow was observed, but since the control line was reached, it did not result in false results. It is therefore always important to check that the milk flow has reached the control line before interpreting the results.

This diminished flow was also observed with blank ewes' milk, but all blank milk remained negative. Blank goats' milk samples also always obtained negative results. Doped ewes' and goats' milk gave positive results.

The AnticFast® Beta-lactams Rapid Test Kit can therefore be used to analyse UHT milk, sterilized milk (if ampicillin and amoxicillin are detected at MRL), thawed milk and reconstituted milk powder. The test can also be used in ewes' and goats' milk.

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

	Ratio			Results	
	mean	min	max	N neg	N pos
Blank milk					
normal raw cows' milk = reference	1.8723	1.7769	2.0153	10	0
UHT	2.1956	1.9396	2.3563	10	0
sterilized	2.3954	2.1376	2.6677	10	0
milk powder	1.7947	1.4937	2.1034	10	0
thawed milk	2.0246	1.8895	2.2017	10	0
goats' (blank n=20)	1.6239	1.4491	1.9194	20	0
ewes' (blank n=20)*	1.7450	1.3191	2.1211	20	0
Milk with benzylpenicillin at 1 µg/kg					
normal raw cows' milk = reference	0.5646	0.5241	0.6253	0	10
UHT	0.7770	0.6843	0.8898	0	10
sterilized	0.8582	0.6862	1.0357	2	8
milk powder*	0.5387	0.3341	0.7311	0	10
thawed milk	0.4794	0.4423	0.5367	0	10
goats' milk	0.6691	0.5826	0.7172	0	10
ewes' milk	0.6570	0.5206	0.8549	0	10
Milk with cefalonium at 1 µg/kg					
normal raw cows' milk = reference	0.8341	0.7298	0.9282	0	10
UHT	1.0111	0.8917	1.1460	5	5
sterilized	1.0908	0.8976	1.4464	7	3
milk powder*	0.6638	0.3953	0.7837	0	10
thawed milk	0.667	0.6203	0.7279	0	10
goats' milk	0.7480	0.6271	0.8535	0	10
ewes' milk	0.7871	0.6036	0.8610	0	10

Notes: *: decreased flow possible; min: minimum; max: maximum. N neg: number of negative results, N pos: number of positive results.

6.5. Stability of reagents – daily control samples

Methods and material:

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

- Blank milk (antibiotic-free raw milk)
- Raw milk doped with 1 µg/kg of benzylpenicillin
- Raw milk doped with 1 µg/kg of cefalonium

Each day, also a negative and positive control (lotnumber and expiry dates equal to the kit reagents) 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.

Results:

The results of the daily control samples and negative and positive control samples are presented in Figures 7 and 8. A summary is provided in Table 15.

Discussion:

Very stable ratio values were obtained for daily control samples with the AnticFast® Beta-lactams 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 doped with a concentration of 1 µg/kg of benzylpenicillin and the milk samples doped with a concentration of 1 µg/kg of cefalonium always resulted in positive results. The negative and positive controls inserted in the kit always gave correct results.

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

Standard	Ratio			
	mean	min	max	s _r
Blank milk	1.8742	1.6587	2.2782	0.13
Milk doped with benzylpenicillin at 1 µg/kg	0.3870	0.1404	0.7326	0.17
Milk doped with cefalonium at 1 µg/kg	0.5209	0.1393	0.7934	0.17
Negative control included in the test kit	1.4402	1.2762	1.5429	0.06
Positive control included in the test kit	0.1036	0.0912	0.1174	0.01

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

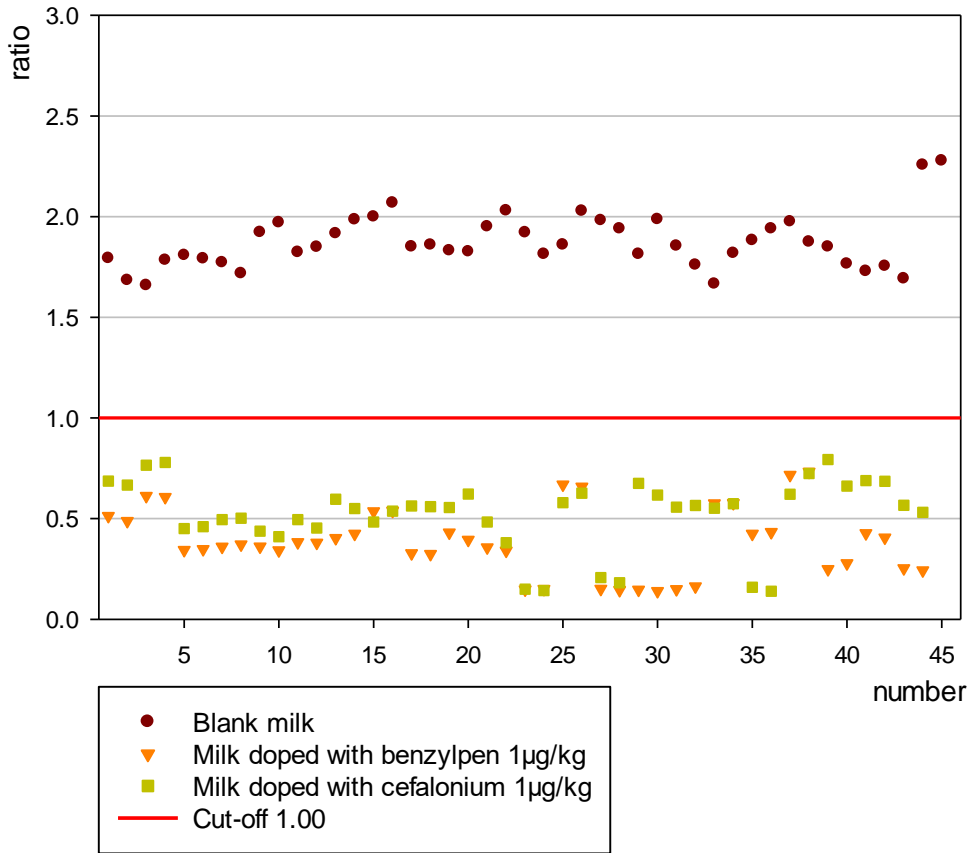


Fig. 7. AnticFast® Beta-lactams Rapid Test Kit results (ratio) for the blank control samples and for doped control samples with benzylpenicillin at 1 µg/kg or doped with cefalonium at 1 µg/kg.

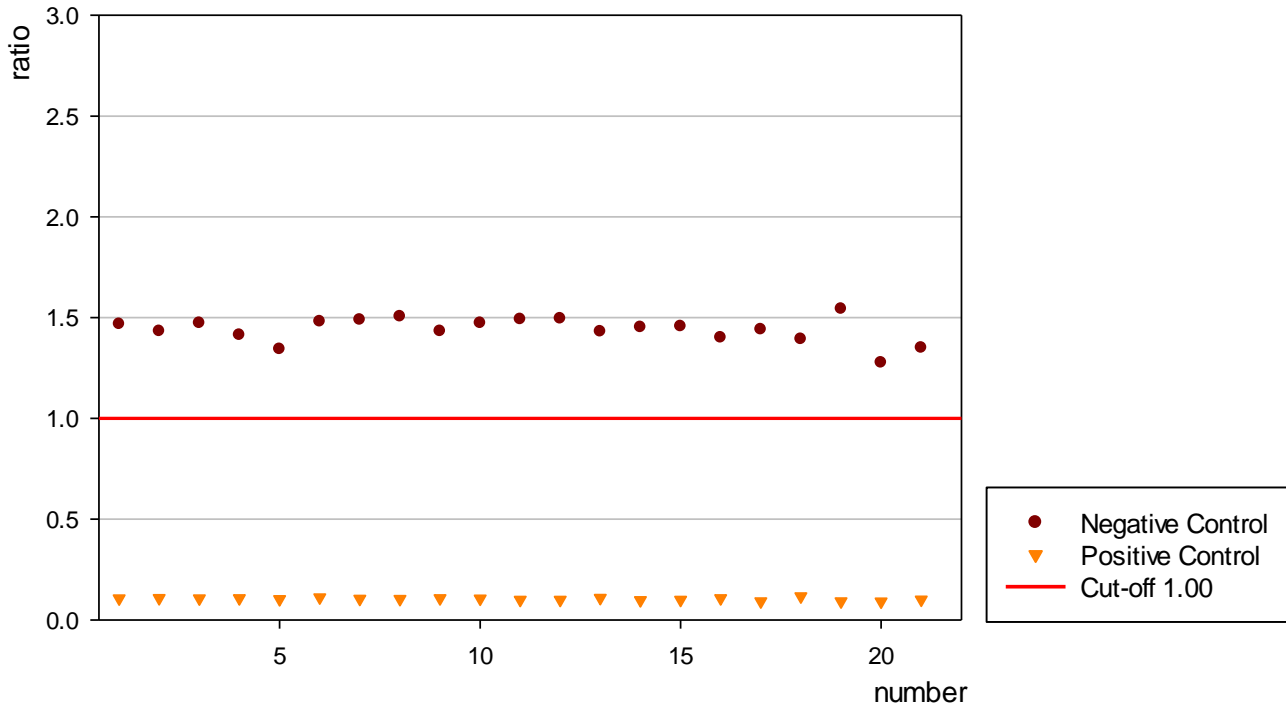


Fig. 8. AnticFast® Beta-lactams Rapid Test Kit results (ratio) for the negative and positive

controls inserted in the kit.

7. Reliability of the instrumentation

Consulting previous results on the reader is difficult as the ID code doesn't change. 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 organizes 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 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	Benzympenicillin	4	4
E	Chlortetracycline	100	100
F	Cefalexin	100	100
G	Blank	-	-
H	Ampicillin	4	4

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

	Visual reading AnticFast® Beta-lactams Rapid Test Kit								Instrumental reading (ratio + result) AnticFast® Beta-lactams 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	
ILVO	-	+	+	+	-	-	-	+	2.1695 NEG	0.3604 POS	0.1648 POS	0.1794 POS	2.0194 NEG	2.5291 NEG	1.9860 NEG	0.5588 POS	20200815G 15/08/2021 BMZ6000 Portable Strip Reader

Note: 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 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 Rapid Test Kit. This is in line with the detection capability reported by Meizheng Bio-Tech for cefalexin (> MRL).

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

Negative results were obtained for the blank milk (sample G) and for the milk samples doped with antibiotics that are supposed to give a negative result. So, there were no false positive results with AnticFast® Beta-lactams Rapid Test Kit.

9. Final conclusion

Results of this validation show that the AnticFast® Beta-lactams 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). With the improved reagents (lot 20210920G (expiration date 20/09/2022) and lot 20211012G (expiration date 12/10/22)), a β-lactams can be detected at least in 95% of the replicates at their respective MRL except for desfuroylceftiofur, and cefalexin.

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.

The test is suitable for other types of milk, such as UHT milk, sterilized milk, thawed milk and reconstituted milk powder (assuming detection of ampicillin and amoxicillin in heat-treated milk). AnticFast® Beta-lactams Rapid Test Kit could also be used to screen ewes' and goats' milk. It is however recommended to check the milk flow on the test strip before interpreting the results.

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