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Validation report
Reagen® Beta-Lactam Strip Test Kit
(RNS92004)
(Reagen® Inc., San Diego, CA)

April 23, 2024

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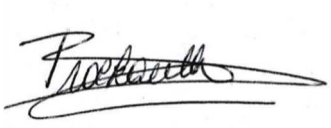

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1. Introduction

Reagen® Beta-Lactam Strip Test Kit (Reagen® Inc., San Diego, CA) (RNS92004) is a qualitative two-step 3+5 minutes rapid lateral flow assay designed to detect beta-lactam antibiotic residues in raw commingled cows' milk.

This test is validated at ILVO-T&V (Technology & Food Science Unit of the Flanders research institute for agriculture, fisheries and food, Melle, BE) according to ISO Technical Specification 23758 | IDF RM 251 (ISO/IDF, 2021), Commission Implementing Regulation 2021/808 and to the EURL Guidance document on screening method validation (*Anon*., 2023). The following analytical parameters were checked: detection capability, test specificity, rate of false positives, repeatability of test and reader and test robustness. Furthermore, the suitability of Reagen® Beta-Lactam Strip Test Kit to screen goats' milk was evaluated. The test will also be included in an interlaboratory study organised by ILVO in April 2024 (results not yet known).

2. Test procedure

Test preparation

Using scissors, carefully cut and remove the numbers of wells and test strips appropriate for the number of samples that will be tested. All reagents should be at ambient temperature (25 ± 5°C) before use. Ensure that unused wells remain firmly capped and re-store the remaining components at 4 °C. Allow the milk sample to reach ambient temperature (the ideal sample temperature is 20-25°C). Shake the milk vigorously to ensure milk sample homogeneity and ensure that the milk has no precipitation or is clotted nor has any abnormal sensory traits. Also the use of colostrum should be avoided.

Test procedure

Step 1: Transfer 200 µL of the milk sample to a microwell with lyophilized gold and mix the sample well with the reagents by pipetting up and down about 10 times, while avoiding bubbles. The sample should turn to a uniform pink color.
Step 2: Incubate the sample for 3 min at 40 ± 2°C in any incubator.
Step 3: After 3 min, insert the bottom of the test strip into the well containing the milk sample. The strip should be inserted as such that the arrows are pointing down. Be sure the strip is oriented vertically (not leaning to the side) and is inserted all the way to the bottom of the well.
Step 4: After 5 min, remove the strip and place it on a horizontal surface with the unmarked side facing up.

For the test lines following counts: Negative: If the test line is stronger than the control line, the milk sample contains no antibiotics or contains antibiotics at lower level than the detection limits. Positive: test line is weaker than (less intense) or equal to the control line, the milk sample contains antibiotics above or equal to the detection limits.

2.1 Configuration of Reagen® Beta-Lactam Strip Test Kit.

The configuration of Reagen® Beta-Lactam Strip Test Kit is shown in Figure 1.

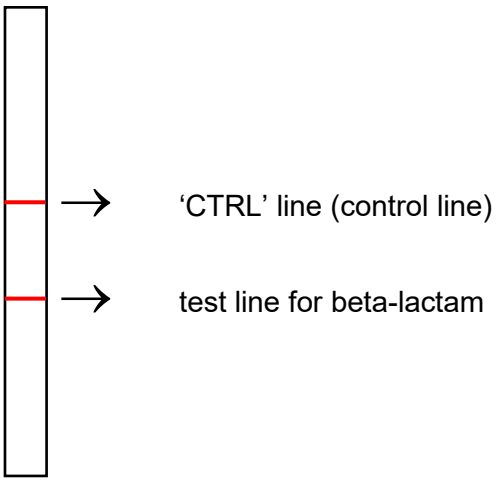

Fig. 1. Configuration of a Reagen® Beta-Lactam Strip Test Kit.

2.2. Instrumental interpretation of the test

The RNE 90324 Test strip reader (Reagen®) 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.

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. RNE 90324 Test strip reader for instrumental reading.

2.3 Visual interpretation of the test

Visual reading of Reagen® Beta-Lactam Strip Test Kit is also possible. The intensity of the test line is compared to the intensity of the reference (i.e. control) line. Negative: test line is darker than the control line; Positive: test line is lighter than or equal to control line, the milk sample contains antibiotics above the detection limits. The interpretation is shown in Figure 3. Visual reading was not checked in this validation study.

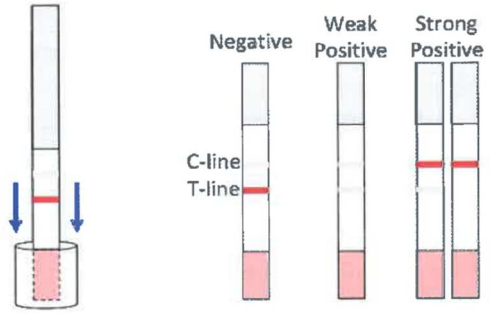

Fig. 3. Visual interpretation of the color formation on Reagen® Beta-Lactam Strip Test Kit (*Anon*., 2024).

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. The milk was 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). Milk of at least 4 animals is commingled and is considered as a sample of standard blank matrix, free from antimicrobial residues and beta-lactamase.

At least four such samples are used for the determination of the detection capability when testing 20 replicates. If 40 or 60 replicates need to be tested to determine the detection capability, minimum eight or twelve different blank milk samples are used, respectively.

The detection capability of Reagen® Beta-Lactam Strip 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 TS 23758 | IDF RM 251 (ISO/IDF, 2021). 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.

- Tested concentration ≤0.5 MRL: 20 times
- Tested concentration >0.5 – <0.9 MRL: 40 times
- Tested concentration ≥0.9 – ≤1.0 MRL: 60 times
- Tested concentration >MRL: 20 times

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

Concentration (in µg/kg)	Increment (in µg/kg)
<1	0.2
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 (fresh and frozen) – twice
- blank raw milk spiked with benzylpenicillin at 1.6 µg/kg – twice
- blank raw milk spiked with cefazolin at 20 µg/kg – twice

These standards will also be used for the robustness analyses.

Detection capability tests were performed with three different lot numbers of reagents: Oct182023 (exp. date 17-10-2024), Nov182023 (exp. date 17-11-2024) and Dec182023 (exp. date 17-12-2024) following the manufacturers' 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 RNE 90324 Test strip reader and software version 1.22 191030. The cut-off value is 1.00 (>1.00: negative; ≤1.00: positive). All results (reader values) were collected in a database.

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

Table 3. Standard material used in this validations study.

Active Compound	Origin	Product number	Lot number	Solvent
Amoxicillin	Dr. Ehrenstorfer	DRE-C10242500	G1193514	ACN/H_2O 50/50*
Ampicillin	Dr. Ehrenstorfer	DRE-C10243080	G1276647	ACN/H_2O 50/50*
Benzylpenicillin	Dr. Ehrenstorfer	DRE-C15935000	G1367278	H_2O
Cefacetrile	HPC Standards	679543	812729	ACN/H_2O 50/50*
Cefalexin	Dr. Ehrenstorfer	DRE-C11064000	G1152231	ACN/H_2O 50/50*
Cefalonium	LGC Mikromol	MM3169.00	1244778	ACN/H_2O 50/50*
Cefapirin	Dr. Ehrenstorfer	DRE-C11064071	G1339721	H_2O
Cefazolin	Dr. Ehrenstorfer	DRE-C11064100	G1339724	H_2O
Cefoperazone	Dr. Ehrenstorfer	DRE-C11064300	G1135405 / G1408543	ACN/H_2O 50/50
Cefquinome	Dr. Ehrenstorfer	DRE-C11064700	G1382333	ACN/H_2O 50/50*
Ceftiofur	Dr. Ehrenstorfer	DRE-C11065000	G1338950	25%FA + H_2O
Chloramphenicol	Dr. Ehrenstorfer	DRE-C11120000	G974527	MeOH
Clavulanic acid	Dr. Ehrenstorfer	DRE-C11668545	G1342649	H_2O
Cloxacillin	Dr. Ehrenstorfer	DRE-C11692100	G1067932	H_2O
Colistin	Sigma-Aldrich/Supelco	PHR1605	LRAC9149	H_2O
Dapsone	Dr. Ehrenstorfer	DRE-C11963000	G1143440	MeOH
Desacetyl cephapirin	HPC Standards	682120	822055	ACN/H_2O 50/50*
Desfuroyl ceftiofur	TRC	D289980	4-SUB-46-2	MeOH
Dicloxacillin	Dr. Ehrenstorfer	DRE-C12560500	G1226254	H_2O
Enrofloxacin	Dr. Ehrenstorfer	DRE-C13170000	G1116914	MeOH
Erythromycin A	Sigma-Aldrich	46256	BCCB8689	MeOH
Lincomycin	Sigma-Aldrich/Supelco	PHR1657	LRAC9565	H_2O
Nafcillin	Dr. Ehrenstorfer	DRE-C15402400	1335481	H_2O
Neomycin	Sigma-Aldrich/Supelco	PHR1491	LRAD3724	H_2O
Oxacillin	Dr. Ehrenstorfer	DRE-C15755100	G1262742	H_2O
Oxytetracycline	Dr. Ehrenstorfer	DRE-C15820000	G1125763	H_2O
Phenoxymethylpenicillin	Dr. Ehrenstorfer	DRE-C15935010	G1069505	H_2O
Sulfadiazine	Dr. Ehrenstorfer	DRE-C16990500	G1390658	1M NaOH
Trimethoprim	Dr. Ehrenstorfer	DRE-C17875000	G1127755	MeOH

Note: *dissolved in a small amount of ACN/H_2O 50/50, and further diluted with H_2O; FA: formic acid.

Results:

A summary of Reagen® Beta-Lactam Strip Test Kit detection capabilities is given in Table 4.

Discussion:

The Reagen® Beta-Lactam Strip Test Kit is capable to detect residues of all beta-lactams with a MRL in milk (EU-Regulation 37/2010 and amendments). All beta-lactams can be detected at least in 95% of the samples at their respective MRL except for desfuroylceftiofur (CCβ=400 µg/kg, MRL=100 µg/kg) and cefalexin (CCβ=225 µg/kg, MRL=100 µg/kg).
It is worth noting that for desfuroylceftiofur, a very unstable metabolite of ceftiofur in solution, it was noticed that the solvent might have an impact on the 95% detection capability on the Reagen® Beta-Lactam Strip Test Kit, therefore further testing will be performed.

Table 4. Detection capability (in µg/kg) of Reagen® Beta-Lactam Strip Test Kit (Reagen, San Diego, CA) in raw bovine milk with instrumental reading (RNE 90324 Test strip reader and software version 1.22 191030) 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 samples, respectively.

Antibiotic Group/ antibiotic	EU MRL (µg/kg)	Detection capability (µg/kg)
Penicillins		
Benzylpenicillin	4	2
Ampicillin	4	2
Amoxicillin	4	2
Oxacillin	30	3
Cloxacillin	30	2
Dicloxacillin	30	3
Nafcillin	30	5
Phenoxymethylpenicillin	-- (25[a])	2
Cefalosporins		
Ceftiofur	100[b]	35
Desfuroylceftiofur	100[b]	**>100[d]**
Cefquinome	20	4
Cefazolin	50	18
Cephapirin	60[c]	3
Desacetylcephapirin	60[c]	7
Cefacetrile	125	8
Cefoperazone	50	3
Cefalexin	100	**225**
Cefalonium	20	2

Notes: MRL: Maximum Residue Limit. Commission Regulation (EU) No 37/2010 and amendments (actual situation);
[a]: No MRL in milk, MRL based on Commission Implementing Regulation (EU) 2018/470.
[b]: The MRL of 100 µg/kg is applied on the sum of all residues retaining the β-lactam structure expressed as desfuroylceftiofur;
[c]: The MRL of 60 µg/kg in milk is applied on the sum of cephapirin and desacetylcephapirin;
[d]: Under further investigation.

4. Test selectivity and rate of false positive results

4.1. Test selectivity

Methods and Materials:

The selectivity of the different test lines of Reagen® Beta-Lactam Strip Test Kit was tested by analysing milk spiked with β-lactam compounds and by analysing milk spiked with compounds belonging to different antibiotic or chemotherapeutic families (1 per family) to check the selectivity of the β-lactam test line. Raw milk was spiked at a high concentration (100×MRL or 100×RPA (Reference point for action (Regulation 2019/1871) /MMPR (Minimum Method Performance Requirement (MMPR) (*Anon.*,

2020)) 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), enrofloxacin (quinolones), colistin (polymyxins), chloramphenicol (amphenicols), erythromycin (macrolides), lincomycin (lincosamides), clavulanic acid (β-lactamase inhibitors), trimethoprim (diamino pyrimidine derivatives) and dapsone (others chemotherapeutics).

More details about the standard material can be found in Table 3.

Results:

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

Table 5. Ratios obtained for different substances doped at high concentration in raw milk and tested with Reagen® Beta-Lactam Strip Test Kit.

Family	Compound	MRL (µg/kg)	Conc. spiked in milk (µg/kg)	Beta-lactam channel	
				Ratio	Result
Tetracyclines	oxytetracycline	100[a]	10.000	5.504	-
Sulfonamides	sulfadiazine	100[b]	10.000	5.330	-
Aminoglycosides	neomycin B	1500	150.000	2.831	-
Quinolones	enrofloxacin	100[c]	10.000	5.004	-
Polymyxins	colistin	50	5.000	8.022	-
Amphenicols	chloramphenicol	0.15[d]	15	7.206	-
Macrolides	erythromycin A	40	4.000	5.561	-
Lincosamides	lincomycin	150	15.000	6.872	-
β-lactamase inhibitors	clavulanic acid	200	20.000	0.022	+
Diamino pyrimidine derivatives	trimethoprim	50	5.000	5.817	-
Others (sulfones)	dapsone	5[e]	500	5.623	-

Notes: MRL: Maximum Residue Limit, Regulation (EC) No 470/2009 of the European Parliament and of the Council and Commission Regulation (EU) No 37/2010 and amendments (current situation). Conc.: concentration.

[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, RPA or reference point for action for chloramphenicol from 28 November 2022 on, Commission Regulation (EU) 2019/1871;
[e]: Prohibited substance, Minimum Method Performance Requirement (MMPR) (*Anon.*, 2020).

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). The 95% detection capability for clavulanic acid was determined at 350 µg/kg.

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

4.2. Test for false-positive results

Methods and materials:

300 blank farm and 300 tanker load milk samples were tested with Reagen® Beta-Lactam Strip Test Kit. In case of positive results, the samples were tested with other microbiological and receptor screening tests to determine whether it is a false-positive result. When it remained inconclusive, the sample was analyzed with LC-MS/MS.

Results and discussion:

All 300 farm and 300 tanker load milk samples tested negative for β-lactams on Reagen® Beta-Lactam Strip Test Kit. So in total no false positive results (0%) are obtained upon 600 samples on all channels. The results are summarized in Table 6.

Table 6. Beta-lactams results for blank farm and tanker milk samples.

	Farm milk (n=300)	Tanker milk (n=300)
	Ratio	**Ratio**
mean	5.723	5.867
min	3.484	2.578
max	9.281	9.986
SD	0.84	0.87
CV%	14.65	14.85

Notes: mean: mean ratio; min: lowest ratio; max: highest ratio; SD ; 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 the β-lactam 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 Reagen® Beta-Lactam Strip Test Kit results are summarized in Table 7.

Table 7. Repeatability of the reader.

Repeatability of the reader	Ratio		
	Mean ratio	s_r	CV%
Blank milk	3.878	0.04	1.14
Low pos	0.803	0.02	3.04
High pos	0.382	0.01	2.71

Notes: mean: mean ratio on the respective channel; 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; the highest variance value was 3.04%.

5.2 Repeatability of the test

Methods and Materials:

Ten blank, 10 low positive samples and 10 high positive samples for the beta-lactam channel were analysed in duplicate. For the spiked samples, any compound found positive could be used.

Results:

The results of the repeatability of Reagen® Beta-Lactam Strip Test Kit are summarized in Table 8.

Table 8. Repeatability of the test.

Repeatability of the test	Ratio		
	Mean ratio	s_r	CV%
Blank milk	5.832	1.38	23.62
Low pos	0.843	0.06	7.23
High pos	0.457	0.09	19.90

Notes: mean: mean ratio on the respective channel; s_r: standard deviation of repeatability; CV(%): relative standard deviation.

Discussion:

The repeatability of the test was good, low standard deviation values were obtained. The highest variance value of 23.62 % is still acceptable since this variance is noted for blank samples with a mean ratio of 5.832. This variation is due to one couple with ratio values 5.087 and 10.310. Also the variance of 19.90% is still acceptable since this is for high positive (low ratio values (mean = 0.457)) values far away from the cut-off value.

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, small changes to the protocol were introduced concerning the timing of the incubation steps, incubation temperature, delay in reading, milk volume and removal of the filter pad.

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 step in the protocol was changed. The normal incubation is 3+5 minutes.

Methods and Materials:

Blank and spiked milk samples containing benzylpenicillin at 1.6 µg/kg or containing cefazolin at 20 µg/kg were analysed (4 samples) with a test protocol with incubation timings different from the test protocol (reference = 3+5 minutes).

Results:

The results of the influence of the length of the incubation step on Reagen® Beta-Lactam Strip Test Kit results are summarized in Table 9.

Discussion:

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

Table 9. Impact of the length of the incubation steps on the Reagen® Beta-Lactam Strip Test Kit results (ratio).

Length of incubation steps	Ratio		
	Blank milk	Milk spiked with benzylpenicillin at 1.6 µg/kg	Milk spiked with cefazolin at 20 µg/kg
2 minutes 45 seconds + 4 minutes 30 seconds			
mean	5.824	0.058	0.754
min	4.926	0.027	0.660
max	6.942	0.077	0.828
2 minutes 45 seconds + 5 minutes			
mean	4.532	0.032	0.609
min	4.295	0.014	0.539
max	4.937	0.053	0.677
2 minutes 45 seconds + 5 minutes 30 seconds			
mean	4.786	0.025	0.568
min	4.509	0.013	0.515
max	5.246	0.034	0.661
3 minutes + 4 minutes 30 seconds			
mean	5.265	0.066	0.623
min	4.853	0.049	0.580
max	6.124	0.087	0.665
3 minutes + 5 minutes (REF)			
mean	5.673	0.144	0.486
min	5.209	0.028	0.449
max	6.493	0.254	0.565
3 minutes + 5 minutes 30 seconds			
mean	5.163	0.038	0.556
min	4.319	0.018	0.490
max	6.230	0.059	0.604
3 minutes 15 seconds + 4 minutes 30 seconds			
mean	5.695	0.026	0.565
min	4.691	0.015	0.459
max	6.931	0.036	0.660
3 minutes 15 seconds + 5 minutes			
mean	5.522	0.017	0.470
min	5.337	0.007	0.398
max	5.704	0.028	0.518
3 minutes 15 seconds + 5 minutes 30 seconds			
mean	4.947	0.021	0.495
min	4.588	0.000	0.443
max	5.392	0.041	0.523

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

6.1.2. Temperature of the incubation

Methods and Materials:

Blank and spiked milk samples containing benzylpenicillin at 1.6 µg/kg or containing cefazolin at 20 µg/kg were analysed (4 replicates) with a test protocol with an incubation at different incubation temperatures (38, 40 (reference) and 42°C).

Results:

The results of the temperature of the incubation step on the Reagen® Beta-Lactam Strip Test Kit results are summarized in Table 10.

Table 10. Ratios obtained when testing blank and spiked milk samples with Reagen® Beta-Lactam Strip Test Kit at 38, 40 (reference) and 42°C, respectively.

	Incubation temperature		
Sample	**38°C**	**40°C (REF)**	**42°C**
Blank milk			
mean	5.281	5.673	5.216
min	4.745	5.209	4.808
max	6.071	6.493	5.653
Milk spiked with benzylpenicillin at 1.6 µg/kg			
mean	0.036	0.144	0.035
min	0.015	0.028	0.026
max	0.049	0.254	0.047
Milk spiked with cefazolin at 20 µg/kg			
mean	0.571	0.486	0.506
min	0.484	0.449	0.470
max	0.633	0.565	0.545

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

Discussion:

Variations in the incubation temperature did not significantly impact results; all negative results remained negative and all positive results stayed positive.

6.1.3. Delay of reading

Methods and Materials:

Blank and spiked milk samples containing benzylpenicillin at 1.6 µg/kg or containing cefazolin at 20 µg/kg were analysed (4 samples) with a test protocol with a delay of reading after 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 as soon as possible and no later than 5 minutes after incubation.

Results:

The results of the influence of the delay of reading are summarized in Table 11.

Discussion:

Delay in reading the test strips did not significantly impact the interpretation of the results; all negative results remained negative and all other positive results stayed positive. For blanks, the ratio very slightly lowers a bit with delay of reading, but this has no impact and for spiked samples stable ratios are obtained.

Table 11. Ratios obtained when testing blank and spiked milk samples and reading Reagen® Beta-Lactam Strip Test Kit strips directly after incubation or with a delay of 5 or 10 minutes respectively.

	Delay of reading		
Sample	**No delay (REF)**	**5 min**	**10 min**
Blank milk			
mean	5.673	5.797	5.600
min	5.209	5.486	5.313
max	6.493	6.276	5.871
Milk spiked with benzylpenicillin at 1.6 µg/kg			
mean	0.144	0.157	0.152
min	0.028	0.049	0.044
max	0.254	0.261	0.235
Milk spiked with cefazolin at 20 µg/kg			
mean	0.486	0.515	0.499
min	0.449	0.483	0.447
max	0.565	0.547	0.547

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

6.1.4. Volume of the milk

Methods and Materials:

Blank and spiked milk samples containing benzylpenicillin at 1.6 µg/kg or containing cefazolin at 20 µg/kg were analysed (4 samples) 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 12.

Discussion:

A volume of milk differing 20 µl (10%) from the prescribed volume of 200 µl did not significantly impact the test results on Reagen® Beta-Lactam Strip Test Kit; the negative results remained negative and positive results stayed positive. It is worth noting that for milk spiked with 1.6 µg/kg of benzylpenicillin, the ratios become slightly more positive when increasing the milk volume.

Table 12. Ratios obtained when testing different volumes (180, 200 and 220 µl, respectively) of milk with Reagen® Beta-Lactam Strip Test Kit.

	Volume of milk		
Sample	**180 µl**	**200 µl (REF)**	**220 µl**
Blank milk			
mean	4.919	5.673	5.681
min	4.466	5.209	5.207
max	5.938	6.493	6.151
Milk spiked with benzylpenicillin at 1.6 µg/kg			
mean	0.207	0.144	0.031
min	0.092	0.028	0.019
max	0.275	0.254	0.043
Milk spiked with cefazolin at 20 µg/kg			
mean	0.642	0.486	0.597
min	0.579	0.449	0.536
max	0.703	0.565	0.679

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

6.1.5. Removal of the filter pad

Methods and Materials:

Blank and spiked milk samples containing benzylpenicillin at 1.6 µg/kg or containing cefazolin at 20 µg/kg were analysed (4 samples) with a test protocol with and without removal of the filter pad.

Results:

The results of the influence of the not removing of the filter pad are summarized in Table 13.

Discussion:

No differences in results are obtained when not removing the filter pad.

Table 13. Ratios obtained with Reagen® Beta-Lactam Strip Test Kit when removing (=reference) or not removing the filter pad before reading.

	Removal of sample pad	
Sample	**With pad**	**Removal of pad (REF)**
Blank milk		
mean	5.858	5.673
min	5.390	5.209
max	6.789	6.493
Milk with benzylpenicillin at 1.6 µg/kg		
mean	0.141	0.144
min	0.000	0.028
max	0.253	0.254
Milk with cefazolin at 20 µg/kg		
mean	0.466	0.486
min	0.420	0.449
max	0.524	0.565

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 (4 samples) with milk of 20°C (= reference) and of 1-4°C in order to check if the milk temperature is influencing Reagen® Beta-Lactam Strip Test Kit results. Besides blank milk also spiked milk samples containing benzylpenicillin at 1.6 µg/kg or containing cefazolin at 20 µg/kg were used.

Results:

The results of the impact of the milk temperature are summarized in Table 14.

Discussion:

The milk temperature (1-4°C) did not significantly impact the Reagen® Beta-Lactam Strip Test Kit results. Blank milk always resulted in negative results and for spiked milk samples positive results on the respective channels were obtained. It is worth noting that for milk spiked with cefazolin at 20 µg/kg, slightly higher ratios are obtained for cold milk (1-4°C), but the results remain clearly positive.

Table 14. Impact of the milk temperature on the Reagen® Beta-Lactam Strip Test Kit result.

	Temperature of milk	
Sample	**1-4°C**	**20°C (REF)**
Blank milk		
mean	5.479	5.673
min	5.067	5.209
max	6.134	6.493
Milk spiked with benzylpenicillin at 1.6 µg/kg		
mean	0.049	0.144
min	0.035	0.028
max	0.061	0.254
Milk spiked with cefazolin at 20 µg/kg		
mean	0.714	0.486
min	0.628	0.449
max	0.784	0.565

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

6.3. Milk quality and milk composition influences

Methods and Materials:

Somatic cell count

Normal milk samples and milk samples with a high somatic cell count (>10^6 per ml) were analysed and the ratios obtained were compared in order to study the impact of the somatic cell count on the Reagen® Beta-Lactam Strip Test Kit result. The milk samples with a high number of somatic cells were selected at the milk control station based on Fossomatic 7 (FOSS, Hillerød. DK) measurements.

Total bacterial count

Normal milk samples and milk samples with a high total bacterial count (TBC >2.0x10^6 CFU per ml) were analysed and the ratios obtained were compared in order to study the impact of the total bacterial count on the Reagen® Beta-Lactam Strip 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, ES) on Plate count agar plates after 3 days incubation at 30°C.

Fat content

Normal milk samples and milk samples with a low (≤2.07 g per 100 ml) or a high (≥6.40 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 Reagen® Beta-Lactam Strip Test Kit result. The milk samples with a low and high fat content were natural milk samples with a low and a high fat content selected at the milk control station based on infrared spectroscopic results with a MilcoScan 7 (FOSS, Hillerød, DK).

Protein content

Normal milk samples and milk samples with a low (≤2.97 g per 100 ml) or a high (≥4.04 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 Reagen® Beta-Lactam Strip 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 7 (FOSS, Hillerød, DK).

pH

Milk samples with a normal pH (6.7 - 6.9) and milk samples with a low pH (6.0) or a high pH (7.5) were analysed and the ratios obtained were compared in order to study the impact of the milk pH on the Reagen® Beta-Lactam Strip Test Kit result. The low and high pH milk samples were prepared by adding 1 M HCl or 1 M NaOH. Respectively, to milk with normal pH. Afterwards the pH of the milk was brought exactly to 6.0 and 7.5 by fine-tuning with 0.1 M HCl and/or 0.1 M NaOH.

Lactation stage

Milk samples from an early lactation (<30 days after calving but no colostrum milk) were analysed and the ratios obtained were compared in order to study the impact of the lactation stage on the Reagen® Beta-Lactam Strip Test Kit result.

Milk samples from a late lactation stage (>270 days after calving) were analysed and the ratios obtained were compared in order to study the impact of the lactation stage on the Reagen® Beta-Lactam Strip Test Kit result.

Results:

With respect to the impact of the milk quality (high somatic cell count, high total bacterial count) and composition (fat and protein content, pH and lactation stage), the mean, the highest and lowest reader value for each milk type are given in Figures 4 to 6 and Table 15.

The legend for the different situations in figures 4 to 6.

1. normal milk = reference
2. SCC >10^6/ml
3. TBC >2.0x10^6
4. low fat ≤2.07 g/100 ml
5. high fat ≥6.40 g/100 ml
6. low protein ≤2.97 g/100 ml
7. high protein ≥4.04 g/100 ml
8. Low pH (pH = 6.0)
9. High pH (pH = 7.5)
10. Early lactation
11. Late Lactation

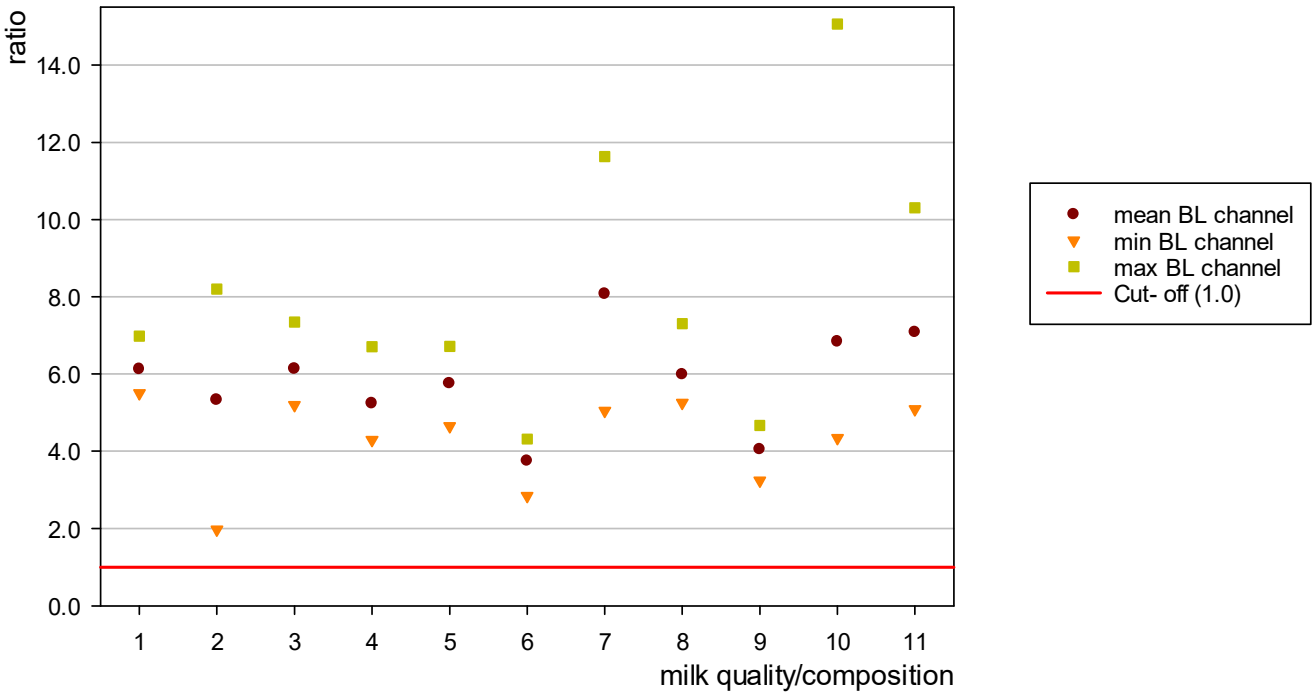

Fig. 4. Results for blank milk, 10 samples. BL: β-lactam channel.

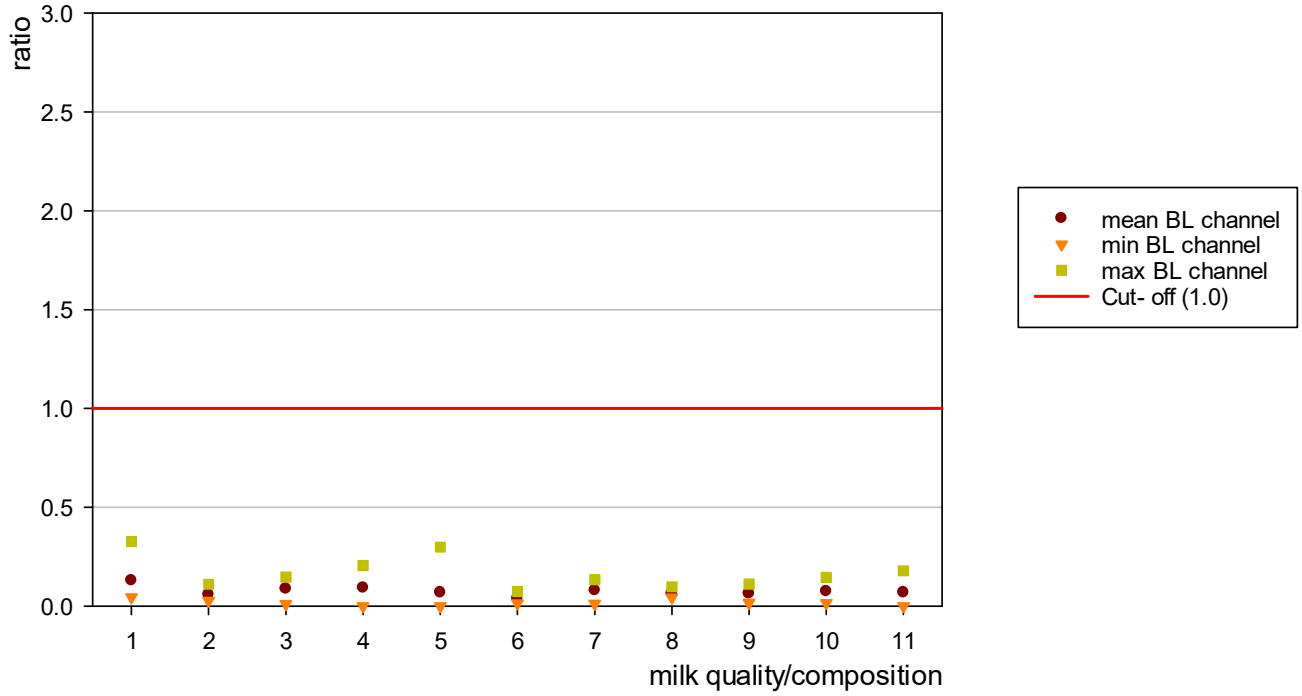

Fig. 5. Results for milk spiked with benzylpenicillin at 1.6 µg/kg, 10 samples. BL: β-lactam channel.

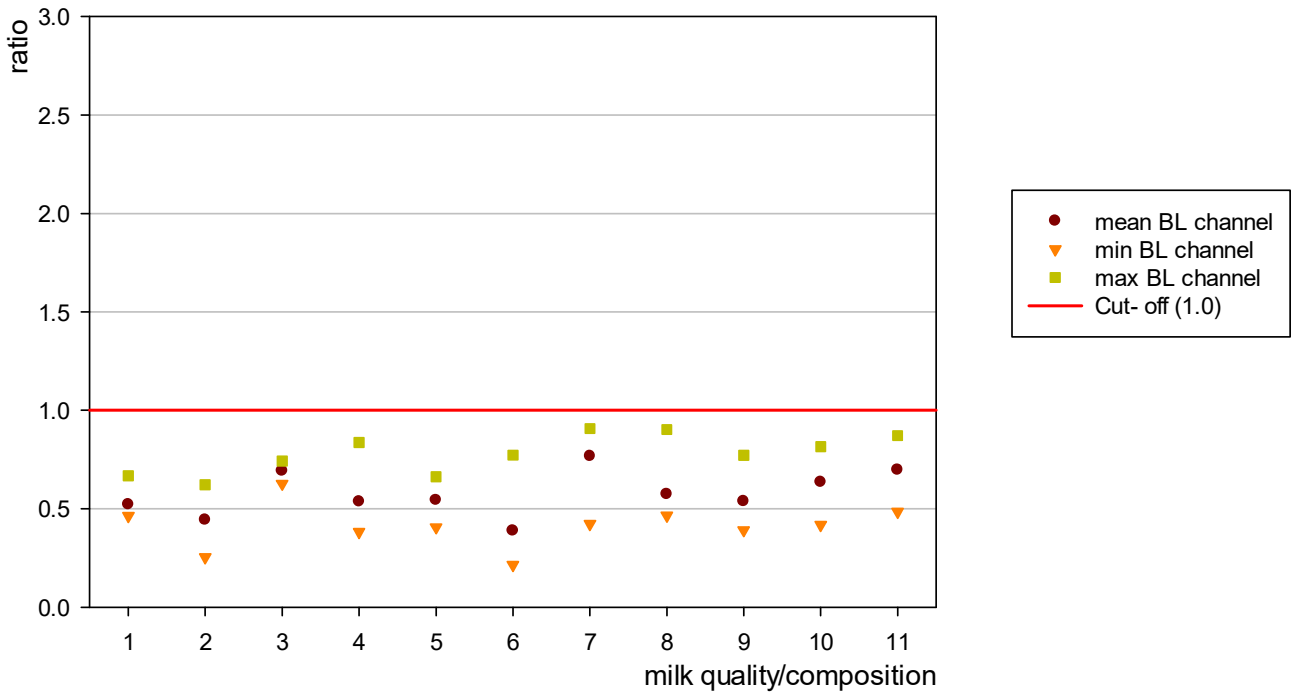

Fig. 6. Results for milk spiked with cefazolin at 20 µg/kg, 10 samples. BL: β-lactam channel.

Discussion:

The milk quality and composition had very little influence on the performance of the Reagen® Beta-Lactam Strip Test Kit. No false positives were obtained with the blank milk, and all spiked samples obtained positive results.

For blank milk it is worth noting that very high ratios can be obtained for milk with a high protein content, and early and late lactation milk. While lower ratios can be obtained for milk with a high somatic cell count, milk with a low protein content or milk with a high pH.

It is also worth noting that 5 positive results were obtained with Reagen® Beta-Lactam Strip Test Kit while testing the special milk types. These samples were further analysed and were found true positives for beta-lactam antibiotics. So it can be concluded that the kit is able to detect beta-lactam residues in incurred milk.

Table 15. Reagen® Beta-Lactam Strip Test Kit results for blank and spiked normal raw cows' milk and for blank and spiked milk of special quality or composition (10 replicates).

	Ratio		
	mean	**min**	**max**
Blank raw cows' milk			
normal milk = reference	6.121	5.508	6.986
SCC >10^6 cells/ml	5.324	1.977	8.220
TBC >2.0x10^6 cfu/ml	6.133	5.197	7.351
low fat ≤2.07g/100 ml	5.237	4.294	6.705
high fat ≥6.40 g/100 ml	5.750	4.648	6.714
low protein ≤2.97g/100 ml	3.751	2.839	4.315
high protein ≥4.04 g/100 ml	8.076	5.051	11.638
low pH (pH = 6.0)	5.985	5.253	7.303
high pH (pH = 7.5)	4.047	3.242	4.665
early lactation	6.840	4.341	15.065
late lactation	7.083	5.091	10.306
Milk spiked with benzylpenicillin at 1.6 µg/kg			
normal milk = reference	0.129	0.045	0.326
SCC >10^6 cells/ml	0.057	0.027	0.110
TBC >2.0x10^6 cfu/ml	0.087	0.010	0.146
	0.092	0.000	0.205
high fat ≥6.40 g/100 ml	0.068	0.000	0.298
low protein ≤2.97g/100 ml	0.037	0.014	0.074
high protein ≥4.04 g/100 ml	0.079	0.012	0.133
low pH (pH = 6.0)	0.063	0.043	0.097
high pH (pH = 7.5)	0.063	0.017	0.111
early lactation	0.074	0.016	0.145
late lactation	0.068	0.000	0.178
Milk spiked with cefazolin at 20 µg/kg			
normal milk = reference	0.521	0.463	0.667
SCC >10^6 cells/ml	0.443	0.254	0.622
TBC >2.0x10^6 cfu/ml	0.691	0.627	0.742
low fat ≤2.07g/100 ml	0.536	0.383	0.836
high fat ≥6.40 g/100 ml	0.543	0.406	0.662
low protein ≤2.97g/100 ml	0.388	0.215	0.772
high protein ≥4.04 g/100 ml	0.766	0.423	0.907
low pH (pH = 6.0)	0.574	0.465	0.902
high pH (pH = 7.5)	0.537	0.391	0.771
early lactation	0.635	0.418	0.816
late lactation	0.697	0.485	0.871

Notes: min: minimum; max: maximum ; SCC : somatic cell count; TBC: total bacteria count; cfu: colony forming unit.

6.4. Type of milk and animal origin influences

Methods and Materials:

Raw goats' milk samples were analysed to determine if Reagen® Beta-Lactam Strip Test Kit is a suitable test for this milk type. Both individual and tank raw goats' milk was used.

Results:

With respect to the impact of goats' milk, the mean, the highest and lowest reader value for the milk type is given in Figures 7 to 9 and Table 15.

The legend for the different situations in figures 7 to 9.

1. Reference: normal raw milk
2. Goats' milk

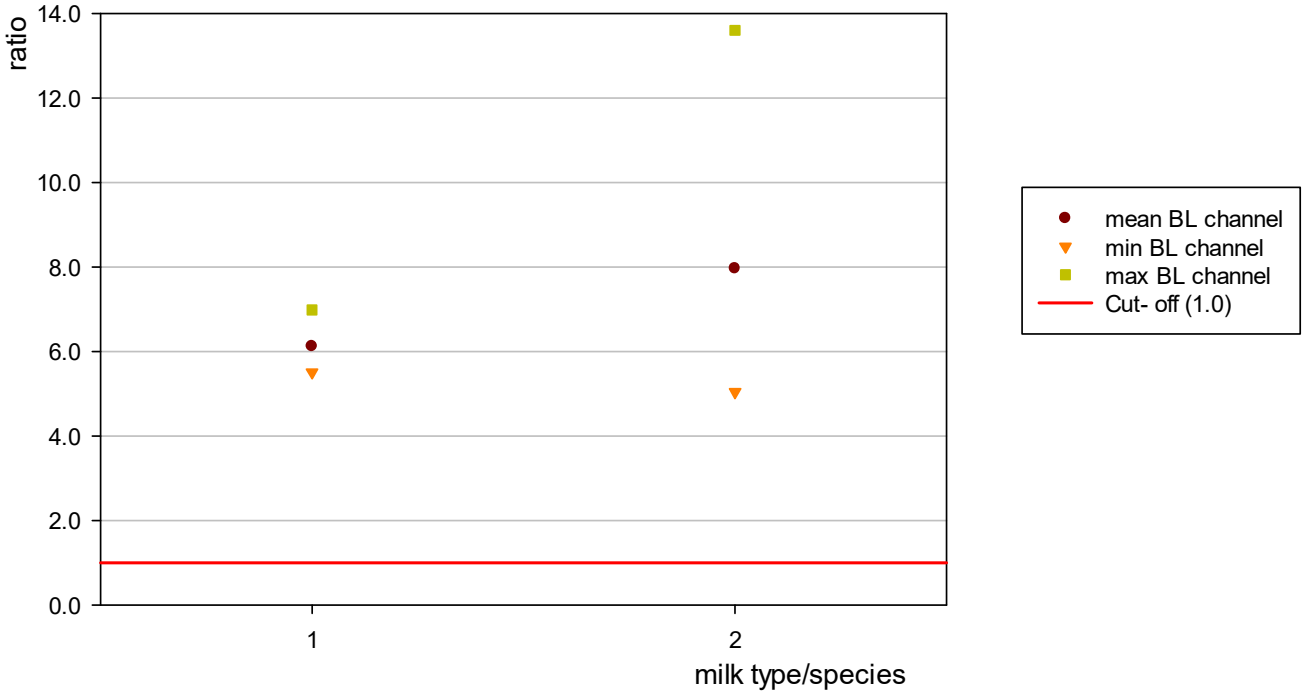

Fig. 7. Results for blank milk, 10 samples. BL: β-lactam channel.

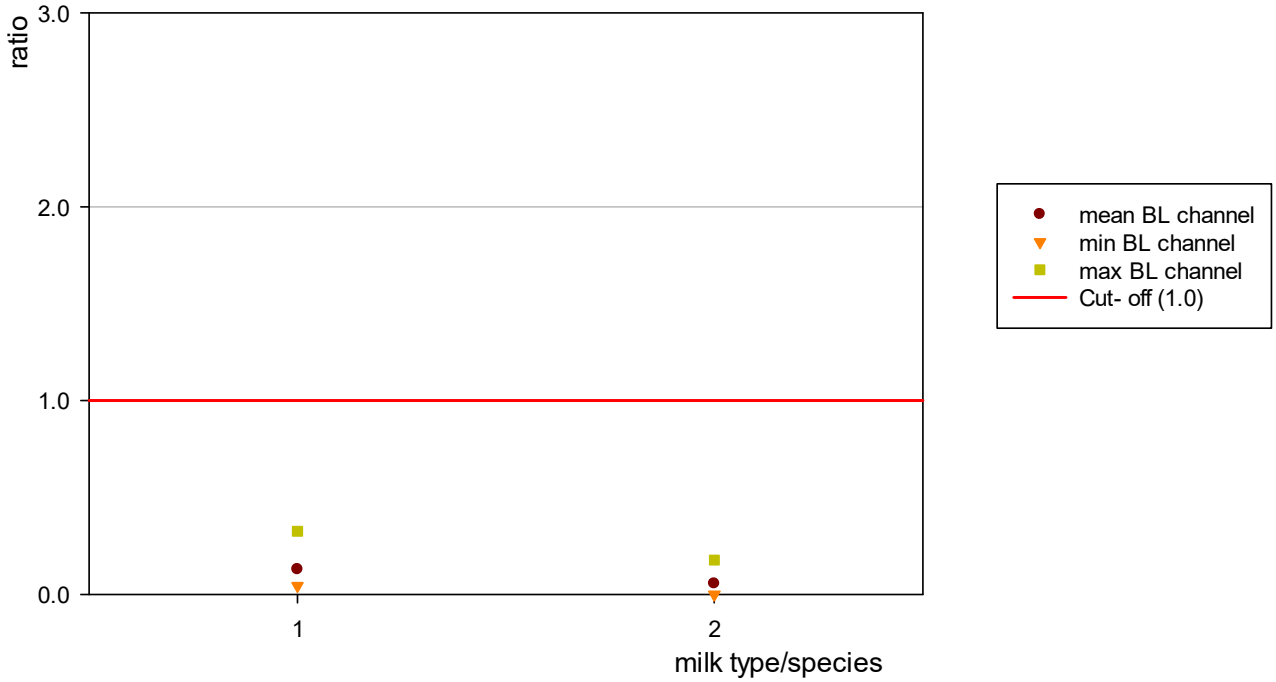

Fig. 8. Results for milk spiked with benzylpenicillin at 1.6 µg/kg, 10 samples. BL: β-lactam channel.

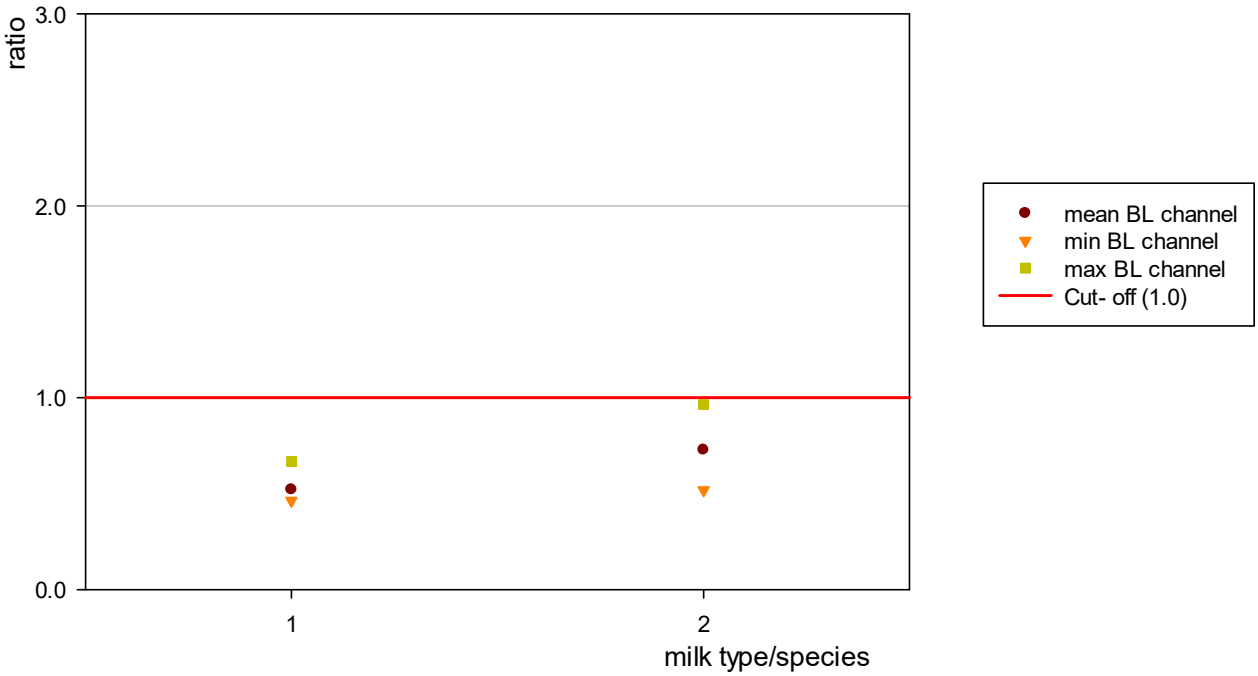

Fig. 9. Results for milk spiked with cefazolin at 20 µg/kg, 10 samples. BL: β-lactam channel.

Table 16. Reagen® Beta-Lactam Strip Test Kit results for blank and spiked normal raw cows' milk and for blank and spiked goats' milk (10 samples, except for 20 samples of blank goats' milk).

	Ratio		
	mean	**min**	**max**
Blank raw cows' milk			
normal milk = reference	6.121	5.508	6.986
goats' milk	7.967	5.039	13.602
Milk spiked with benzylpenicillin at 1.6 µg/kg			
normal milk = reference	0.129	0.045	0.326
goats' milk	0.055	0.000	0.178
Milk spiked with cefazolin at 20 µg/kg			
normal milk = reference	0.521	0.463	0.667
goats' milk	0.727	0.518	0.964

Notes: min: minimum; max: maximum.

Discussion:

There could be interest to use the Reagen® Beta-Lactam Strip Test Kit, although developed for the testing of raw cows' milk, to test raw goats' milk.

Results show that the Reagen® Beta-Lactam Strip Test Kit can also be used for raw goats' milk. It is worth noting that for blank samples, the highest ratios are obtained in individual goats' milk.

6.5. Stability of reagents - Daily control samples

Methods and material:

The following control samples were analyzed daily with Reagen® Beta-Lactam Strip

Test Kit to check the stability of the reagents and consistency of results:

- blank raw milk free from antimicrobials (fresh and frozen) – twice
- blank raw milk spiked with benzylpenicillin at 1.6 µg/kg – twice
- blank raw milk spiked with cefazolin at 20 µg/kg – twice

Results:

The results of the daily control samples are presented in Figure 10 and 11. A summary is provided in Table 17.

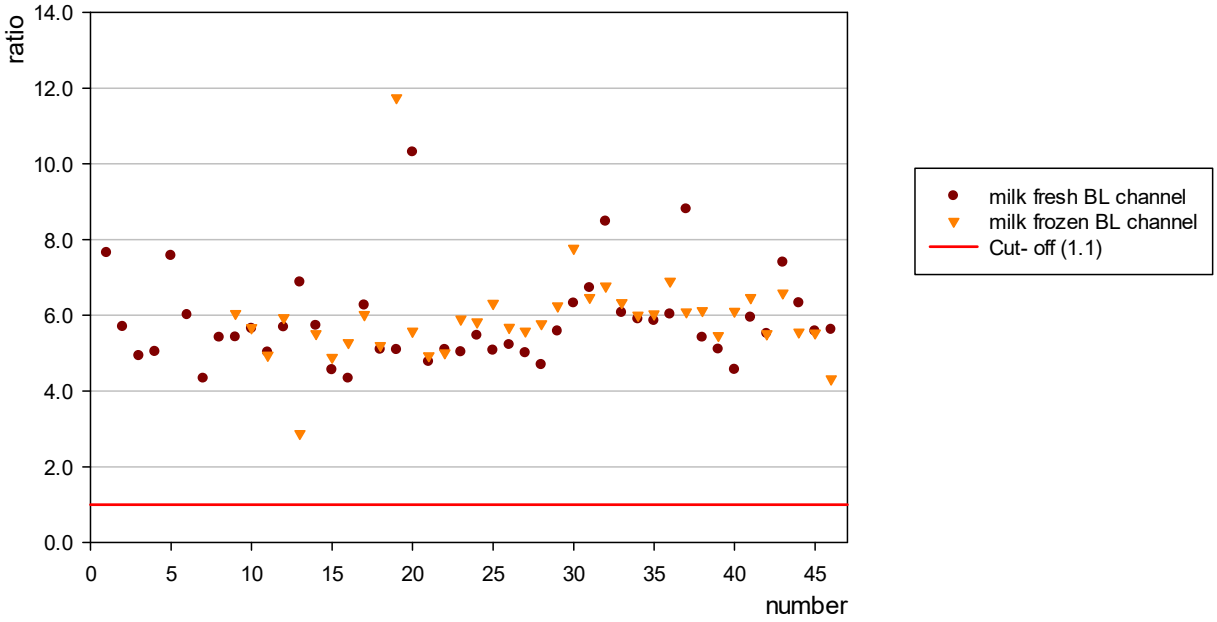

Fig. 10. Reagen® Beta-Lactam Strip Test Kit results (ratio) for the blank control samples (fresh and frozen) of the daily standard. BL: β-lactam channel.

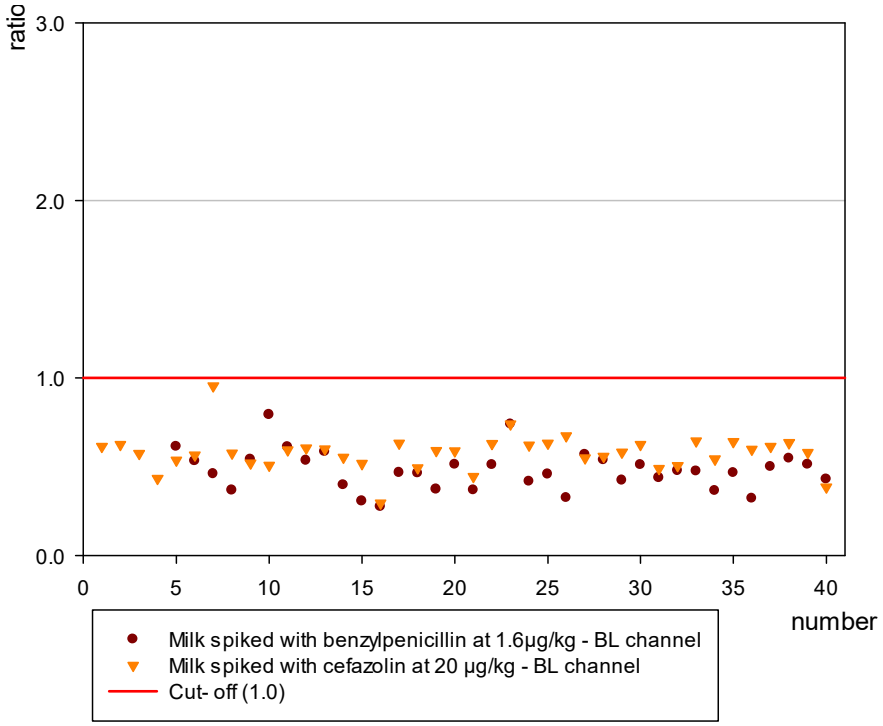

Fig. 11. Reagen® Beta-Lactam Strip Test Kit results (ratio) for the spiked samples with benzylpenicillin at 1.6 µg/kg or with cefazolin at 20 µg/kg of the daily standards. BL: β-lactam.

Table 17. Reagen® Beta-Lactam Strip Test Kit results (ratio values) for the daily standards.

Standard	Ratio			
	mean	min	max	s_r
Blank milk				
Fresh	5.830	4.329	10.310	1.20
Frozen	5.922	2.879	11.745	1.26
Milk spiked with benzylpenicillin at 1.6 µg/kg.				
	0.478	0.275	0.793	0.11
Milk spiked with cefazolin at 20 µg/kg				
	0.578	0.295	0.956	0.10

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

Discussion:

In general stable ratio values were obtained for daily control samples with the Reagen® Beta-Lactam Strip Test Kit reagents over the test period. Always correct values were obtained for the different daily standards. All blank milk standards gave a negative result and all spiked milk samples gave positive results. It is worth noting that the ratio values of milk spiked with 1.6 µg/kg of benzylpenicillin is higher (less positive) than during the rest of the study, but this is most probably due to the fact that the spiked daily control samples have been frozen while all other testing was performed on fresh raw cows' milk.

7. Reliability of the instrumentation

No issues occurred with the instruments during this validation study.

But it is worth noting that when the strip is incorrectly inserted in the reader, no "invalid" is given, but a result is obtained, which might be different from when the strip is inserted correctly. It is therefore important to make sure that a strip is inserted in the correct way.

For use in a laboratory it would be interesting to be able to add a sample ID in the reader.

8. Final conclusion

The validation of Reagen® Beta-Lactam Strip Test Kit (Reagen, San Diego, CA) was executed following ISO Technical specification TS 23758 / IDF RM 251.

The validation results of the Reagen® Beta-Lactam Strip Test Kit show that the test is a fast, simple and reliable highly specific test for screening of raw cows' milk for residues of β-lactam antibiotics (penicillins and cefalosporins) in raw commingled cows' milk (Commission Regulation 37/2010 and amendments). All β-lactams can be detected at least in 95% of the samples at their respective MRL except for desfuroylceftiofur (exact CCβ under investigation) and cefalexin (CCβ=225 µg/kg, MRL=100 µg/kg).

Reagen® Beta-Lactam Strip Test Kit is a highly specific test for detection of beta-lactams in milk. Only clavulanic acid, a β-lactamase inhibitor, gave an (expected) interference at the β-lactam channel with a 95% detection capability of 350 µg/kg.

No false positive results (0%) were obtained when testing 300 blank farm and 300 blank tanker load milk samples and both reader and test repeatability are good.

The test shows to be very robust when changes in the test protocol (length of incubation steps, incubation temperature, delay of reading, milk temperature, volume of milk, removal of sample pad) are introduced. Also the milk quality and composition had no significant influence on the performance of the Reagen® Beta-Lactam Strip Test Kit. And validation results show that the test is also suitable to use for raw goats' milk for the detection of residues of beta-lactam antibiotics.

ACKNOWLEDGEMENT

The authors appreciate the valuable work performed by Caroline Poleyn, Annelies Wachtelaer, Sofie De Schryver and Sophie Veys and thank Reagen® Inc. for kindly providing Reagen® Beta-Lactam Strip Test Kit reagents and MelkControleCentrum Vlaanderen for providing part of the raw cows' milk samples with a special composition or quality and for the MilcoScan 7, Fossomatic 7 and Fossomatic FC measurements.

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