Validation report of
Milksensor® CBTSC10i
(β-lactams & Tetracyclines, Streptomycin & Chloramphenicol)
(Product N° CBTSC10i)

(Mayasan A.S., Istanbul, Turkey)
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1. Introduction

Validation report of Milksensor® CBTSC10i (β-lactams & Tetracyclines, Streptomycin & Chloramphenicol) Test Kit (Mayasan A.S., Istanbul, Turkey). The test is a colloidal gold immunochromatography two-step 10-min (3'+7') rapid lateral flow assay to detect β-lactam, tetracyclines, streptomycine and chloramphenicol antibiotic residues in raw commingled cows' milk.

This new test was validated 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, Commission Implementing Regulation 2021/808 and to the CRL guidelines for the validation of screening methods for residues of veterinary medicines (*Anonymous*, 2010). The following analytical parameters were checked: detection capability, test specificity and test robustness (impact of deviation of the test protocol, impact of the milk composition or milk type). The test was also included in the interlaboratory study organised by ILVO on 22 October 2021.

2. Test procedure

Test preparation
Bring the test kit and samples to room temperature with tubes closed to avoid moisture and sunlight. Prepare the milk sample at room temperature which is fresh and free from precipitation and clotting.
The test can also be used for milk powder. Then the sample should be prepared by diluting 1:9 (10 g of milk powder and 90 ml of water). This was however not tested in this validation, only raw milk was used.

Test procedure
1st step: Turn on the metal incubator and wait until it is stable at 42°C
2nd step: Add 200 µl milk to a microwell and mix well by pipetting up and down 10 times.
3rd step: Place the micro-wells in the incubator, and incubate for 3 minutes at 42°C. During this time place the dipsticks onto the incubator.
4th step: At the end of the first incubation, the dipsticks will fall down and automatically the second incubation will start (7 minutes at 42°C).
5th step: Take out the strip from the well and remove the sample pad at the lower end. Read the results visually or by using the Milksensor® Reader.

For the test lines T1, T2 and T4 (streptomycin, chloramphenicol and tetracycline, respectively) following counts: Negative: 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 the control line or no line appears, the milk sample contains antibiotics above the detection limits.
For test line T3 (β-lactams): Negative: weak or strong test line. Positive: no test line.

2.1 Configuration of the Milksensor® CBTSC10i (β-lactams & Tetracyclines, Streptomycin & Chloramphenicol) Test Kit test strip

The configuration of the Milksensor® CBTSC10i (β-lactams & Tetracyclines, Streptomycin & Chloramphenicol) Test Kit test strip is shown in Figure 1.
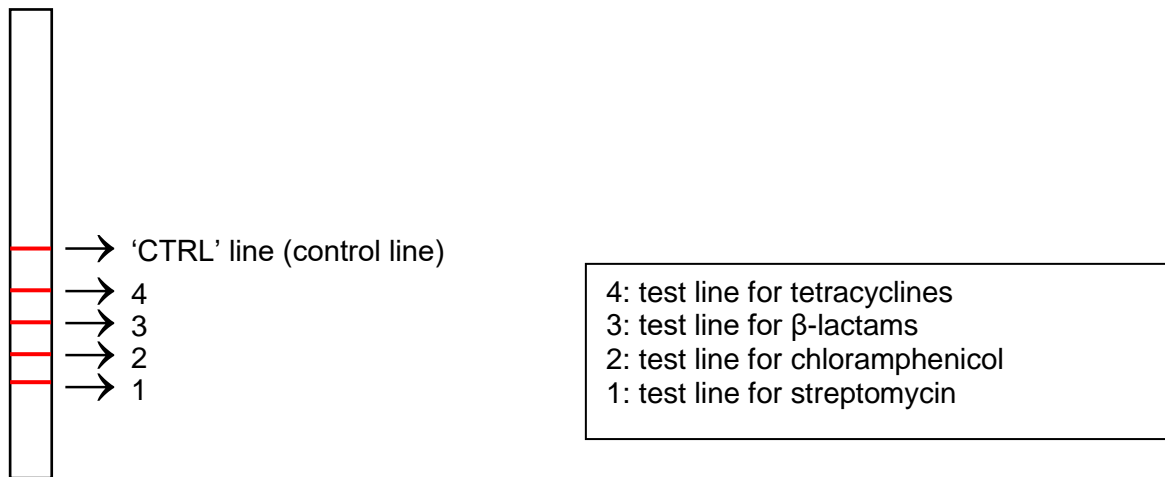
Fig. 1. Configuration of an Milksensor® CBTSC10i (β-lactams & Tetracyclines, Streptomycin & Chloramphenicol) Test Kit test strip.

2.2. Instrumental interpretation of the test

The Milksensor® 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. The ratio cut-off levels for each test line are given in Table 1.

In this validation both 'doubt' and 'positive' are considered as positive.

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

| Ratio				
Beta-lactam channel	Tetracycline channel	Chloramphenicol channel	Streptomycin channel	Interpretation
R≤ 0.17	R≤ 0.75	R≤ 0.95	R≤ 0.7	Positive (+)
0.17<R≤0.25	0.75<R≤0.85	0.95<R≤1.10	0.7<R≤0.85	Doubt (+/-)
R＞0.25	R＞0.85	R＞1.10	R＞0.85	Negative (-)

Fig. 2. Milksensor® incubator (*Anon.*, 2021a) and reader model GIC-S1 (*Anon.,* 2021b) for instrumental reading of the color formation on Milksensor® CBTSC10i (β-lactams & Tetracyclines, Streptomycin & Chloramphenicol) Test Kit.

2.3 Visual interpretation of the test

Visual reading of Milksensor® CBTSC10i (β-lactams & Tetracyclines, Streptomycin & Chloramphenicol) Test Kit is also possible. The intensity of the test line is compared to the intensity of the reference (i.e. control) line.
Color intensity of tetracycline, chloramphenicol or streptomycin line is weaker than control line or no-line appears: tetracycline, chloramphenicol or streptomycin positive, respectively.
No β-lactam line: β-lactam positive; weak color intensity is considered as negative.

Visual reading was not checked in this validation study.
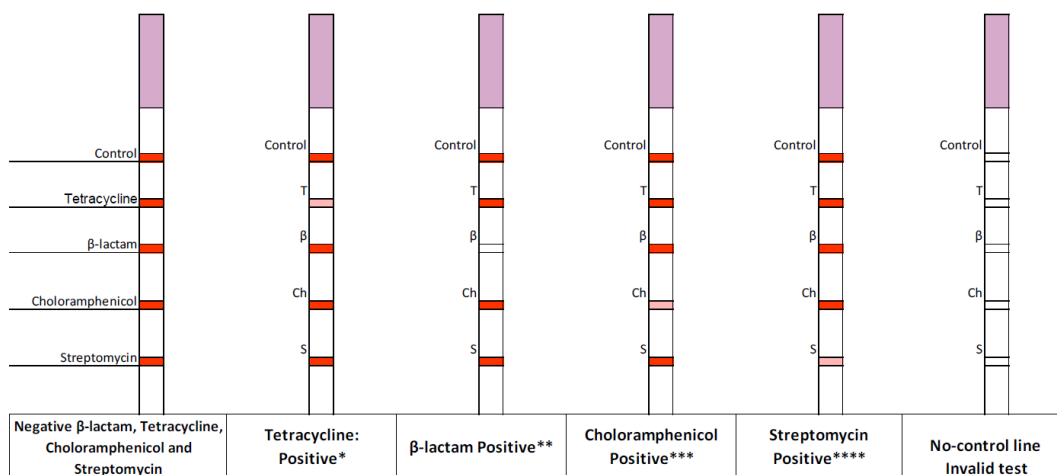
Fig. 3. Visual interpretation of the color formation on Milksensor® CBTSC10i (β-lactams & Tetracyclines, Streptomycin & Chloramphenicol) Test Kit. (*Anon.*, 2021c)

3. Detection capability

Methods and Materials:

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

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 Milksensor® CBTSC10i (β-lactams & Tetracyclines, Streptomycin & Chloramphenicol) Test Kit was determined for a selection of β-lactams, tetracyclines, streptomycin and chloramphenicol mentioned as marker residue in Table 1 of the annex of Commission Regulation (EU) No 37/2010, as well as chloramphenicol. Whereas for the other compounds on the list, at was tested whether they could be detection at their respective MRL level. 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)
1-10	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 2 µg/kg, oxytetracycline at 4 µg/kg streptomycin at 80 µg/kg, and chloramphenicol at 0.3 µg/kg – twice

Detection capability tests were performed with at least 2 different lot numbers of reagents (lot 46MR11 and 46MR12, with as expiration date 09/09/2022 and 10/09/2022, respectively) 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 Milksensor ® reader model GIC-S1. The cut-off value for each test line is given in Table 1. All results (reader values) were collected in a data base.

Table 3. Standard material used in this validations study.

Compound	Origin	Product number	Lot number
4-Epichlortetracycline hydrochloride	LGC Standards	C13175500	1146697
4-Epioxytetracycline	LGC Standards	C13179000	1150794
4-Epi-tetracycline hydrochloride	TRC	T291405	1-EDT-29-1
Amoxicillin trihydrate	LGC Standards	C10242500	G1012320
Ampicillin trihydrate	LGC Standards	C10243080	G1059460
Benzylpenicillin	SIGMA-ALDRICH	46609	BCCB4506
Cefacetrile	HPC Standards	679543	799234
Cefalexin monohydrate	LGC Standards	C11064000	G984299
Cefalonium dihydrate	LGC Standards	MM3169.00	G985467
Cefapirin sodium	SIGMA-ALDRICH	43989	BCCC5208
Cefazolin	SIGMA-ALDRICH.	C0682800	5.0
Cefoperazone sodium	LGC Standards	C11064300	G1135405
Cefquinome sulfate	LGC Standards	C11064700	G1005777
Ceftiofur hydrochloride	TRC	C244700	8-SCC-55-1
Ceftiofur	LGC Standards	C11065000	G1104213
Chloramphenicol	LGC Standards	C11120000	G974527
Chlortetracycline hydrochloride	SIGMA-ALDRICH	46133	BCBT9837
Cloxacillin sodium salt monohydrate	SIGMA-ALDRICH	46140	BCBW1061
Desfuroylceftiofur	TRC	D289980	4-WBZ-137-3
Desacetylcephapirin sodium salt	HPC Standards	682120	801433
Dicloxacillin sodium monohydrate	LGC Standards	C12560500	G1039116
Doxycycline Hyclate	LGC Standards	C13084280	1116543
Nafcillin Sodium monohydrate	LGC Standards	C15402500	G1089700
Oxacillin sodium salt monohydrate	LGC Standards	C15755100	G1019309
Oxytetracycline Hydrochloride	SIGMA-ALDRICH	O5875	057M4020V
Oxytetracycline hydrochloride	SIGMA-ALDRICH	46598	BCBZ6310
Pencillin G sodium salt	SIGMA-ALDRICH	PENNA	045M4815V
Penicillin V potassium salt	SIGMA-ALDRICH	46616	BCBV7003
Tetracycline hydrochloride	SIGMA-ALDRICH N.V.	31741	BCCC9767

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

Table 4. Detection capability (in µg/kg) of Milksensor® CBTSC10i (β-lactams & Tetracyclines, Streptomycin & Chloramphenicol) Test Kit (Mayasan SA., Istanbul, Turkey) in raw bovine milk with instrumental reading (Milksensor® reader model GIC-S1) with cut-off ratio 0.25 for the beta-lactam test line, 0.85 for the tetracycline and streptomycine test lines and 1.10 for the chloramphenicol test line. 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	MR(P)L (µg/kg)	Customs Union MPL (µg/kg)	Detection capability (µg/kg)
Penicillins	Benzylpenicillin	4	4	2
	Ampicillin	4	4	2
	Amoxicillin	4	4	3
	Oxacillin	30		3
	Cloxacillin	30		< MRL*
	Dicloxacillin	30		< MRL*
	Nafcillin	30		8
	Phenoxymethylpenicillin	-- (4)[a]		< MRL*
Cefalosporins	Ceftiofur	100[b]		90
	Desfuroyl ceftiofur	100[b]		**> MRL****
	Cefquinome	20		6
	Cefazolin	50		< MRL*
	Cephapirin	60[c]		5
	Desacetylcephapirin	60[c]		< MRL*
	Cefacetrile	125		14
	Cefoperazone	50		< MRL*
	Cefalexin	100		**> MRL****
	Cefalonium	20		< MRL*
Tetracyclines	Tetracycline	100[d]	10[e]	6
	4-Epimer of tetracycline	100[d]	10[e]	< MRL*
	Oxytetracycline	100[d]	10[e]	3
	4-Epimer of oxytetracycline	100[d]	10[e]	< MRL*
	Chlortetracycline	100[d]	10[e]	20
	4-Epimer of chlortetracycline	100[d]	10[e]	< MRL*
	Doxycycline	--[f]		9
Streptomycins	Streptomycin	200	200	60
	Dihydrostreptomycin	200		< MRL*
Phenicols	Chloramphenicol	0.3[g]	0.3	0.3

Notes:< MRL*: Detection capability below MRL; exact detection capability not tested; **> MRL****: Detection capability above 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 (actual situation). Customs Union MPL: Maximum Permissible Level, Russia-Kazakhstan-Belarus Customs Union (CU), Customs Union Technical Regulation on Milk and Dairy Products. GAIN Report Number: RS1382 of 11/18/2013. 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]: 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]: The MRL of 100 µg/kg in milk is applied on the sum of parent drug and its 4-epimer,

[e]: The MPL of 10 µg/kg in milk is applied on the sum of parent drug and its 4-epimer,

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

[g]: MRPL or Minimum Required Performance Limit, Commission Decision 2003/181/EC.

Results:

A summary of Milksensor® CBTSC10i (β-lactams & Tetracyclines, Streptomycin & Chloramphenicol) Test Kit detection capabilities is given in Table 4.

Discussion:

The Milksensor® CBTSC10i (β-lactams & Tetracyclines, Streptomycin & Chloramphenicol) Test Kit is capable to detect all residues of β-lactams (penicillins and cefalosporins), streptomycins, chloramphenicol and tetracyclines present on the EU-MRL list in milk (Commission Regulation (EU) No 37/2010. For those β-lactams for which the 95% detection capability was determined, all can be detected at least in 95% of the replicates at their respective MRL. For the other β-lactams, testing of two replicates shows that all are detected at their respective MRL concentration except for desfuroylceftiofur and cefalexin.

All tetracyclines can be detected at least in 95% of the replicates at their respective MRL. The detection capability of their 4-epimers was not determined, but they are detectable at MRL level. 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 9 µg/kg on.

Also streptomycin was detected at least in 95% of the replicates at its respective MRL. The 95% detection capability of dihydrostreptomycine was not determined, but testing of three replicates shows that the compound is detected below its MRL.

Chloramphenicol (prohibited compound) can be detected at least in 95% of the replicates from 0.3 µg/kg (= MRPL (Commission Decision 2003/181/EC)).

Regarding the Russian milk standards (Customs Union, 2020), the Milksensor CBTSC10i (β-lactams & Tetracyclines, Streptomycin & Chloramphenicol) Test Kit is capable to detect the required penicillins, tetracyclines, streptomycin and chloramphenicol in raw commingled cows' milk with at least a 95% detection at Customs Union MPL level.

4. Stability of reagents – daily control samples

Methods and material:

The following control samples were analyzed daily (twice) with Milksensor® CBTSC10i (β-lactams & Tetracyclines, Streptomycin & Chloramphenicol) Test Kit to check the stability of the reagents and consistency of results:

- Blank milk (antibiotic-free raw milk)

- Raw milk doped with benzylpenicillin at 2 µg/kg, oxytetracycline at 4 µg/kg streptomycin at 80 µg/kg and chloramphenicol at 0.3 µg/kg.

Also the negative and positive control samples included in the kit were analysed daily (lotnumber and expiry date equal to reagents).
For the negative control, take 200 µl of HPLC-water and put into the negative control microwells. Mix homogenously by pipetting up and down. Then transfer the mixture into a regular microwell and mix homogenously with the reagent.
For the positive control microwells, take 200 µl of negative milk sample and put into the microwell and mix homogenously. Transfer the mixture into a regular microwell and mix homogenously with the reagent.

Results:
The results of the daily control samples are presented in Figure 4 and 5. A summary is provided in Table 5. The results of the daily negative and positive control samples included in the kit are presented in Figure 6 and Table 5.
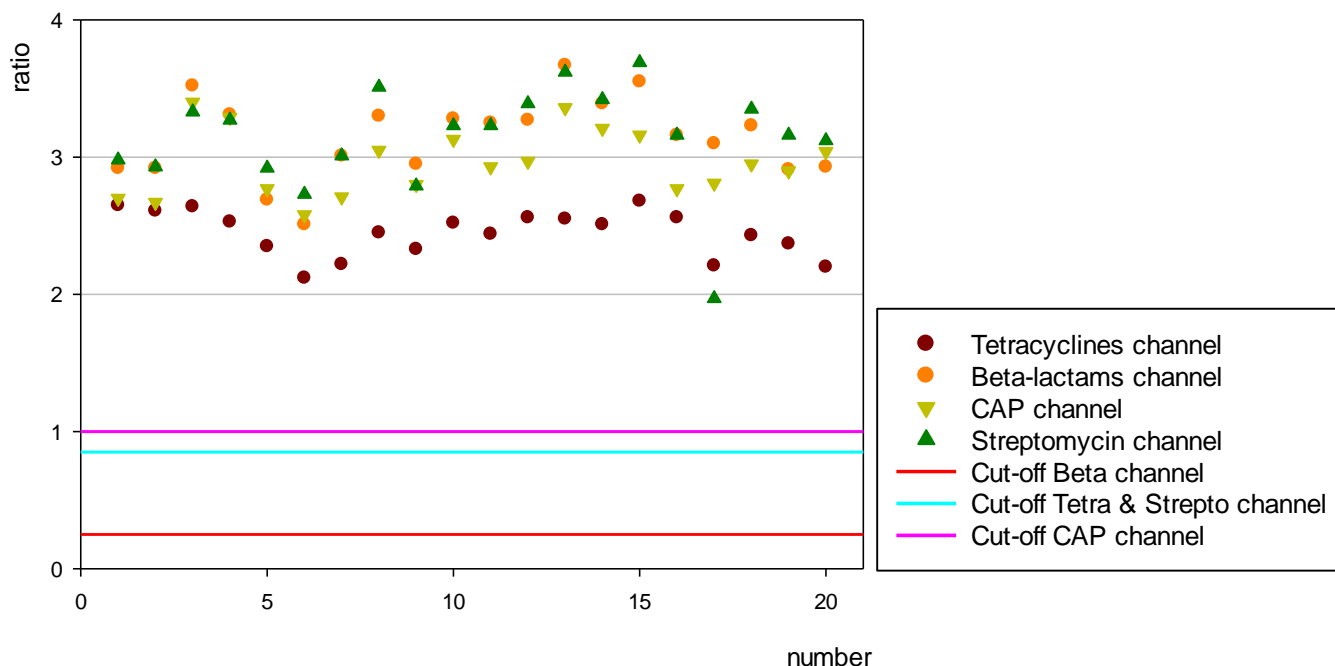
Fig. 4. Milksensor® CBTSC10i (β-lactams & Tetracyclines, Streptomycin & Chloramphenicol) Test Kit results (ratio) for the blank. Beta: β-lactam; Strepto: streptomycin; CAP: chloramphenicol; Tetra: tetracycline.
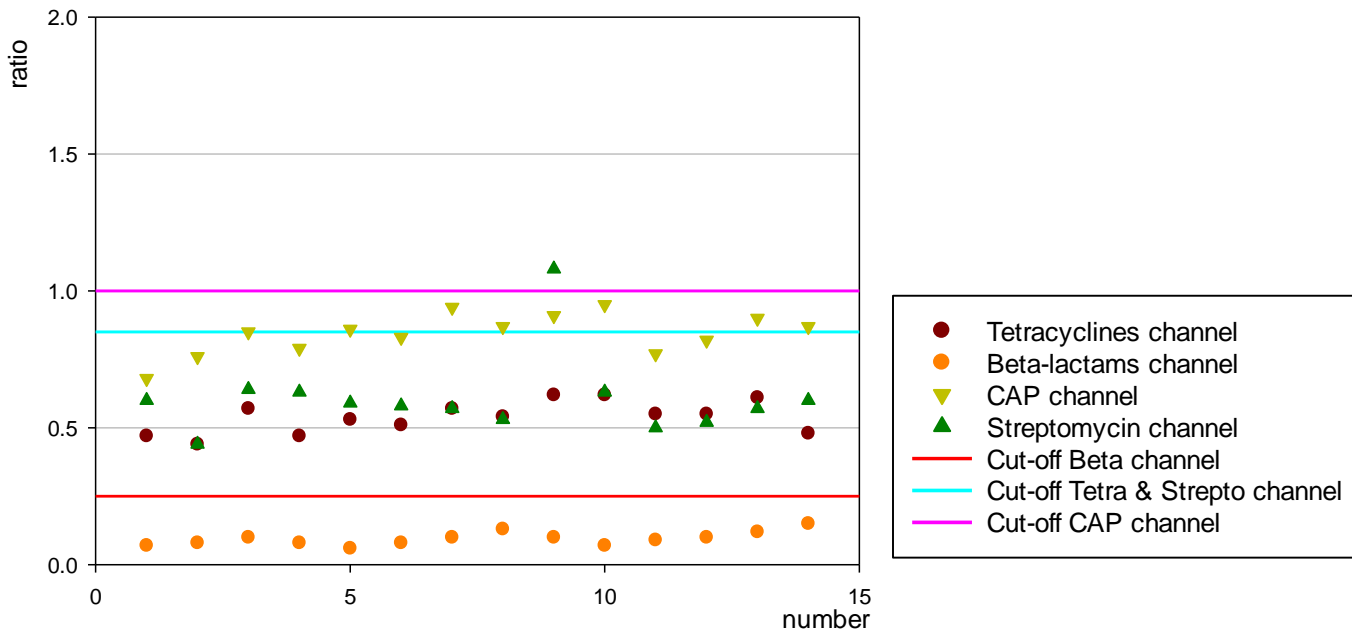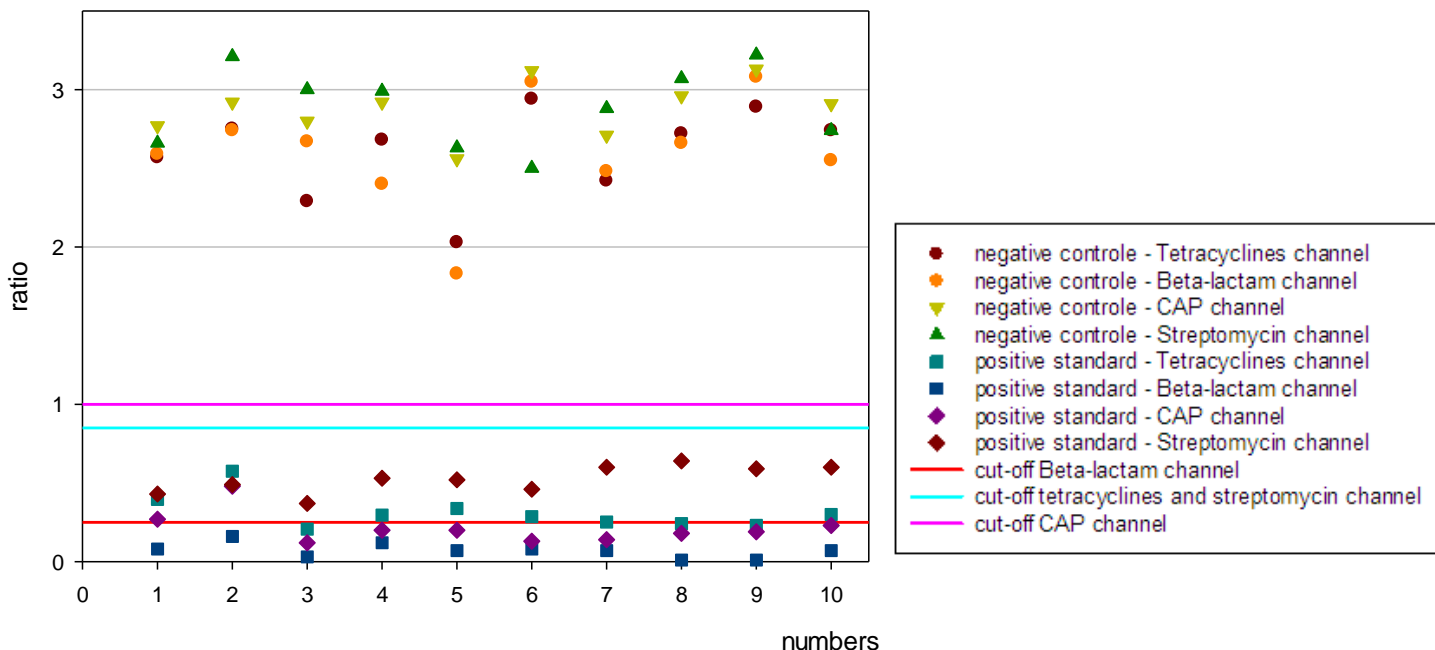
Fig. 5. Milksensor® CBTSC10i (β-lactams & Tetracyclines, Streptomycin & Chloramphenicol) Test Kit results (ratio) for the samples spiked with benzylpenicillin at 2 µg/kg, oxytetracycline at 4 µg/kg streptomycin at 80 µg/kg and chloramphenicol at 0.3 µg/kg. Beta: β-lactam; Strepto: streptomycin; CAP: chloramphenicol; Tetra: tetracycline.

Table 5. Milksensor® CBTSC10i (β-lactams & Tetracyclines, Streptomycin & Chloramphenicol) Test Kit results (ratio values) for the daily standards and negative and positive control samples.

Standard	Tetra-channel				Beta-lactam channel				Chloramphenicol channel				Streptomycin channel			
	mean	min	max	s_r	mean	min	max	s_r	mean	min	max	s_r	mean	min	max	s_r
Daily standard - Blank milk																
	2.45	2.12	2.68	0.16	3.14	2.51	3.67	0.29	2.96	2.58	3.40	0.24	3.14	1.97	3.69	0.38
Daily standard - Milk doped with benzylpenicillin at 2 µg/kg, oxytetracycline at 4 µg/kg, streptomycin at 80 µg/kg and chloramphenicol at 0.3 µg/kg.																
	0.54	0.44	0.62	0.06	0.09	0.06	0.15	0.02	0.84	0.68	0.95	0.07	0.61	0.44	**1.08**	0.15
Controls included in kit																
Negative	2.60	2.03	2.94	0.28	2.60	1.83	3.08	0.35	2.88	2.56	3.13	0.18	2.89	2.50	3.22	0.25
Positive	0.31	0.21	0.57	0.11	0.07	0.01	0.16	0.05	0.21	0.12	0.48	0.10	0.52	0.37	0.64	0.09

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

Fig. 6. Milksensor® CBTSC10i (β-lactams & Tetracyclines, Streptomycin & Chloramphenicol) Test Kit results (ratio) for the negative and positive control samples included in the kit. Beta: β-lactam; Strepto: streptomycin; CAP: chloramphenicol; Tetra: tetracycline.

Discussion:

In general very stable ratio values were obtained for daily control samples with the Milksensor® CBTSC10i (β-lactams & Tetracyclines, Streptomycin & Chloramphenicol) Test Kit reagents over the test period on all tests lines. In general, correct values were obtained for the different daily standards: all blank milk standards gave a negative result on all channels.

For the pooled milk samples doped with benzylpenicillin at 2 µg/kg, oxytetracycline at 4 µg/kg, streptomycin at 80 µg/kg and chloramphenicol at 0.3 µg/kg, all tested positive on all four test channels. One sample tested negative on the streptomycin channel (ratio 1.08), without a clear reason (ccβ = 60 µg/kg).

The daily negative and positive control samples included in the kit gave correct results at all times. Negative control samples were always negative, positive control samples were always positive.

5. Reliability of the instrumentation

- During this validation study, no problems with the reader system not incubator were encountered.
- In the kit insert (*Anon.*, 2021c), interpretation of the results visually is written as final step. With a cut-off value of 0.25 for the beta-lactam test line, 0.85 for the tetracycline and streptomycine test lines and 1.10 for the chloramphenicol test line this is not easy and reliable for positive samples containing beta-lactams close to ccβ concentration.

- When removing the sample pad of the strip people have to take care that no sample pad residues are left behind, as even the littlest residue might give false results.
- The negative test control does not dissolve very well in HPLC-water.

6. Interlaboratory testing

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 October 2021, Milksensor® CBTSC10i (β-lactams & Tetracyclines, Streptomycin & Chloramphenicol) Test Kit was integrated as rapid test.

Results:

Identification of the samples:

Sample	Drug	Conc. (µg/kg)	MRL (µg/kg)
I	Amoxicillin	4	4
J	Blank	-	-
K	Oxytetracycline	100	100
L	Nafcillin	15	4
M	Cloxacillin	30	30
N	Benzylpenicillin	3	4
O	Ceftiofur	20	100
P	Cephapirin	40	60

Table 6. Results of Milksensor® CBTSC10i (β-lactams & Tetracyclines, Streptomycin & Chloramphenicol) Test Kit in the national ring trial of 22 October 2021 (Ooghe & Reybroeck, in preparation).

LAB	Instrumental reading (ratio + result) Milksensor® CBTSC10i								Lot number Expiry date Type of reader
	I	J	K	L	M	N	O	P	
TETRACYCLINES									
ILVO	2.31 NEG	2.72 NEG	**0.09 POS**	2.36 NEG	2.47 NEG	2.24 NEG	2.58 NEG	2.18 NEG	46MR12 10/09/2022 Milksensor®-reader (model GIC-S1)
BETA-LACTAM									
ILVO	**0.08 POS**	3.47 NEG	3.08 NEG	**0.11 POS**	**0.09 POS**	**0.05 POS**	1.85 NEG	**0.08 POS**	
CHLORAMPHENICOL									
ILVO	2.72 NEG	3.21 NEG	2.78 NEG	2.77 NEG	3.06 NEG	2.54 NEG	3.11 NEG	2.74 NEG	
STREPTOMYCINS									
ILVO	2.85 NEG	3.43 NEG	3.02 NEG	2.93 NEG	3.11 NEG	2.99 NEG	3.09 NEG	2.90 NEG	

Notes: The cut-off value of the MilkSensor®-reader is 0.25 for the beta-lactam test line, 0.85 for the tetracycline and streptomycine test lines and 1.10 for the chloramphenicol test line. Milk samples generating a ratio above these cut-off values are considered negative.

Conclusion

Good results were obtained with Milksensor® CBTSC10i.

Except for sample O, spiked with 20 µg/kg of ceftiofur, all other milk samples fortified with β-lactam antibiotics (samples I, L, M, N & P) were screened positive with Milksensor® CBTSC10i. The negative result is in line with the detection capability of Milksensor® CBTSC10i for ceftiofur (ccβ = 90 µg/kg).

The milk sample fortified with 100 µg/kg of oxytetracycline (sample K) was screened positive on the tetracycline test line of Milksensor® CBTSC10i.

Hence, in this ring test amoxicillin, oxytetracycline, nafcillin, cloxacillin, benzylpenicillin and cephapirin are detected at MRL or below with Milksensor® CBTSC10i.

Negative results were obtained for the blank milk (sample J) on all channels and for the milk samples doped with antibiotics that are supposed to give a negative result (on the respective test lines). So, there were no false positive results with Milksensor® CBTSC10i.

7. Final conclusions

Results of this validation show that Milksensor® CBTSC10i (β-lactams & Tetracyclines, Streptomycin & Chloramphenicol) Test Kit is a reliable test for screening of raw cows' milk for residues of β-lactam (penicillins and cephalosporins), tetracycline and streptomycin antibiotics below their MRL (EU-Regulation 37/2010 and amendments) and chloramphenicol at MRPL (Commission Decision 2003/181/EC). Of several compounds the 95% detection capability was not determined (see Table 4), but testing of two replicates indicate that the test is able to detect the compounds at their MRL, except for cefalexin and desfuroyl ceftiofur for which both replicates were not detected at their respective MRL level.

The Milksensor® CBTSC10i (β-lactams & Tetracyclines, Streptomycin & Chloramphenicol) Test Kit is suitable to screen raw cows' milk for residues of β-lactam antibiotics (penicillins and cefalosporins), tetracyclines, streptomycin and chloramphenicol in raw commingled cows' milk at the Russian milk standards (Customs Union, 2020) with at least a 95% detection.

No selectivity/specificity tests, nor robustness tests were performed in this short validation.

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