EVALUATION OF RAPID TESTS FOR THE DETECTION OF β-LACTAM AND TETRACYCLINE ANTIBIOTICS IN MILK

The objective of this study was to evaluate two kits for the detection of β-lactam and tetracycline antibiotics in milk (kit 1: rapid detection of β-lactam and kit 2: rapid detection of β-lactam and tetracycline).
These kits, manufactured by Bioeasy, are commercialised in France by Humeau.
The tested method is based on a strip immuno-chromatographic technique and is applicable to two different matrixes: raw milk or reconstituted dried milk.
The study was performed in 2 steps:

1. **- Determination of the threshold detection of the method for 14 antibiotics (kit 1) or 18 antibiotics (kit2)**
2. **- Determination of the threshold detection and robustness of the method for 1 antibiotic.**

PRINCIPLE OF THE METHOD

This method is based on a strip immuno-chromatographic technique.

Inoculation and incubation:

200µl of sample are introduced, after homogenisation, in a micro-cell containing the reagent. This micro-cell is incubated during 3 minutes at 40 °C ±3 °C. This first step enables the interaction between the potential antibiotics contained in milk and the reagents contained in the micro-cell.
After this first incubation, the strip is introduced in the micro-cell. A second incubation during 3 minutes for the kit 1 and of during 5 minutes for the kit 2 enables the migration of the reactional mix in the strip.

Reading and interpretation of the results:

The reading was visually realised. An automatic reader exists but was not used within this evaluation.

You will find below the graph and the interpretation grid of the test results for the detection of a molecule:

Figure 1: Interpretation of the results

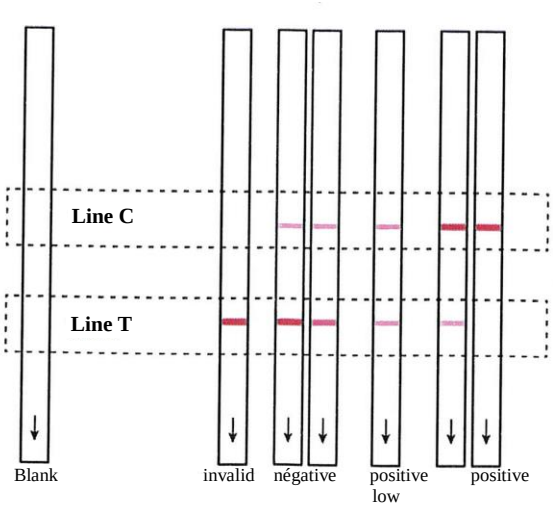

Tableau 1: Interpretation of the results

Comparison of the colour intensity between the T test line and the C control line	Results of analysis	Interpretation of the results
T>C	**NEGATIVE**	The milk sample do not contain antibiotic or contains a quantity of antibiotic lower than the limit value of detection (indicated in the notice).
T=C	**POSITIVE LOW**	The milk sample contains a quantity of antibiotic equal to the detection value (indicated in the notice).
T<C or absence of T line	**POSITIVE**	The milk sample contains a quantity of antibiotic higher than the detection value (indicated in the notice).

MATERIAL AND METHODS

1- Material

For this study, we used:
- metrologically verified 200 µl micropipette
- 3 incubators provided by Humeau for the incubation at 40 °C ±3 °C.

2- Basic raw milk

Each implemented basic raw milk was analysed by the method to evaluate and by the kit of an other provider (Kit C), based on the same strip immuno-chromatographic principle, in order to verify the absence of antibiotic in the basic milk.

2.1 Determination of the threshold detection for 14 antibiotics

For the both evaluated kits, the tests were performed with mix cow raw milk from PDO Comté cheese cooperative. The raw milk implemented was different for each tested antibiotic.

2.2 Determination of the detection threshold and robustness for 1 antibiotic

Two PDO mix raw milks and a non PDO mix raw milk were used to study the variability due to the origin of the milks.

3 - Preparation of the samples

The stock solutions were prepared at 1 mg/ml according to the annex 2 of the AFNOR certification N° NF102[1] requirements, which defines the solvent used for the stock solution, and the temperature and its maximum storage time. The standards were prepared by successive dilutions of the stock solution. Les solutions filles ont été préparées par des dilutions successives de la solution mère. A little volume of the final dilution was added in the basic raw milk to constitute the sample to test.

4 - Protocol

The limits of detection (LOD) forecasted by the provider are in the Humeau notice.
The kit C was used to verify the concentrations of the tested samples.

4.1 Determination of the detection threshold for 14 antibiotics or 18 antibiotics

14 molecules of β-lactam and 4 molecules of tetracycline were tested.
At least 3 concentrations per kit were evaluated for each molecule (announced, a little higher and a little lower LOD). According to the first results obtained, the following concentrations were re-adjusted.
For each antibiotic molecule and for 3 concentrations, 5 samples were analysed in duplicate, many other concentrations were tested on 1 sample in duplicate.
The evaluation was only performed on a batch. It was then not possible to evaluate the inter-batches variability.

4.2 Determination of the detection threshold and robustness for 1 antibiotic

To confirm the results obtained, a second study was performed on a molecule already tested in the first study by varying the batches of the kits and the origin of the implemented milk.
This second study was performed on dicloxacillin, which presented, in the first study, a limit of detection 10 times lower than the announced LOD. Three milk from different origins and two different batches per kit were evaluated.
The results obtained on 10 samples analysed in duplicate by concentration, type of milk and kit enable to determine the capacity of detection (Ccβ) according to the AFNOR certification N° NF102[1] recommendations. Ccβ is the most low content of substance that can be detected, identified and/or measured in a sample, with a β error probability of 5 %.

RESULTS

During the evaluation, any strip has presented invalid tests, the control line was always present.

1 Determination of the detection threshold for 14 antibiotics

The tables below present the totality of the results obtained during the evaluation after the analysis of the 5 samples in duplicate by concentration. The LOD announced by the provider and the maximum regulatory limits (MRL) according to the European Regulation No37/2010 are joined.
The observed LOD column summarises the limits of detection noted in this evaluation. A colour code enables to situate them according the LOD announced by the supplier.

Table 2: Limits of detection obtained for Kit No 1 (detection of β-lactamin)

Family	Antibiotic	LOD announced by the supplier (ppb)*	Observed limit of detection (ppb)*	MRL (ppb)*
ß-lactamin	Penicillin G	**1-1.5**	0.5-1	4
	Ampicillin	**2-4**	0.5-1	4
	Amoxicillin	**2-4**	0.5-1	4
	Oxacillin	**5-7**	2-3	30
	Cloxacillin	**5-7**	2-3	30
	Dicloxacillin	**10-20**	1-2.5	30
	Nafcillin	**20-30**	2.5-5	30
	Cefapirine	**8-10**	6-8	60
	Cefalonium	**6-8**	1-2	20
	Cefazoline	**40-50**	20-30	50
	Cefoperazone	**4-6**	1-2	50
	Cefquinome	**12-18**	2.5-5	20
	Ceftiofur	**80-100**	40-60	100
	Cefacetrile	**25-30**	15-20	125

Table 3: Limits of detection obtained for Kit N° 2 (detection of β-lactamin and tetracycline)

Family	Antibiotic	LOD announced by the supplier (ppb)*	Observed limit of detection (ppb)*	MRL (ppb)*
ß-lactamin	Penicillin G	**1.5-2**	1.5-2	4
	Ampicillin	**3-5**	1-3	4
	Amoxicillin	**3-5**	1-2	4
	Oxacillin	**5-7**	2-3	30
	Cloxacillin	**6-8**	2-3	30
	Dicloxacillin	**10-20**	1-2,5	30
	Nafcillin	**20-30**	2.5-5	30
	Cefapirin	**15-18**	6-8	60
	Cefalonium	**6-8**	2-4	20
	Cefazoline	**40-50**	30-40	50
	Cefoperazone	**4-6**	1-2	50
	Cefquinome	**12-18**	2.5-5	20
	Ceftiofur	**80-100**	40-60	100
	Céfacétrile	**25-30**	15-20	125
Tétracycline	Doxycycline	**30-50**	5-10	ND
	Chloretetracycline	**30-50**	10-20	100
	Oxytetracycline	**30-50**	5-10	100
	Tétracycline	**30-50**	10-20	100

Observed limit = announced LOD
Announced limit/2 < Observed limit < announced limit
Observed limit < announced limit/2

Key for reading the colour code for the interpretation of the differences between the announced LOD and the observed limits of detection

The limit of detection of kit 1 for Penicillin G, Cefapirin and Cefacetrile is between the LOD/2 and the announced LOD. For all the other molecules, the limit of detection observed is very below the announced LOD.

For kit 2, the limit of detection observed for Penicillin G corresponds to the announced LOD. For Ampicillin, Cefazolin and Cefacetrile, the limit of detection observed is between the LOD/2 and the announced LOD. For all the other molecules, the limit of detection observed is very below the announced LOD.

2 Determination of the detection threshold and robustness for 1 antibiotic

Table 4: Results by type of milk and by batch (in % of positive results for n analyses)

KITS	BATCH	Concentration in dicloxacillin (ppb)	Results	PDO milk No 1 n=20	Non-PDO milk n=20	PDO milk No 2 n=20
KIT No1	BATCH A	2.5	% pos	100%	100%	100%
		2	% pos	100%	100%	100%
		1.5	% pos	95%	30%	85%
	BATCH B	2.5	% pos	100%	100%	100%
		2	% pos	100%	100%	100%
		1.5	% pos	90%	30%	85%
KIT No 2	BATCH C	2.5	% pos	100%	100%	100%
		2	% pos	100%	95%	100%
		1.5	% pos	30%	0%	0%
	BATCH D	2.5	% pos	100%	100%	100%
		2	% pos	100%	95%	100%
		1.5	% pos	90%	0%	0%

Ccβ: capacity of detection

The capacity of detection for dicloxacillin is of 2 ppb for all the kits and milk tested, except for the kit No 1 on a milk and a batch with a Ccβ of 1.5 ppb, but the difference is not significant: 100% of positive results / 20 at 2 ppb, against 95% at 1,5 ppb.

Table 5: Results by kit (in % of positive results for n analyses)

Concentration in Dicloxacillin n=120	1.5 ppb	2 ppb	2.5 ppb
KIT No 1 Detection of β-lactam	69%	100%	100%
KIT No 2 Detection of β-lactam and tetracycline	20%	98%	100%

Ccβ: capacity of detection

The capacity of detection for dicloxacilline was globally evaluated at 2 ppb, and is identical for the both kits.

GENERAL CONCLUSION

This detection test for β-lactam and tetracycline antibiotics is quick and convenient to implement, and negative and positive control confirming the performance of the batch are from now on provided with the kit. Its implementation is simple and no particular technical qualification is necessary. The interpretation of the results by visual reading is not difficult thanks the 3 possibilities of results: negative, low positive or positive. Moreover, the lines, after air drying of the strips during 3 minutes, are more marked and it is easier to view the intensity, especially in limit of detection.

For the both kits, detection thresholds lower or equal to the LOD announced by the provider were observed in the first study performed on 10 molecules of β-lactam and 4 molecules of tetracycline.

These results with a determination of Ccβ at 2 ppb for the both kits were confirmed and refined with the second study. The robustness of the method was also demonstrated by this study performed with many batches, different origin milk and different technicians, during several days.

[1]*AFNOR certification N° NF102 : « Protocole de validation des méthodes de détection et de quantification des résidus de médicaments vétérinaires dans les produits agro-alimentaire - Exigences relatives aux études préliminaire et interlaboratores menées par un Laboratoire expert" révision n° 1 : 1er juin 2017*