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Verification of Ballya β-Lactam and Tetracycline rapid test dipsticks for determining inhibitory substance compliance of raw milk samples.

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Executive Summary

BT Sensors Test Kit (Ballya International Limited, Guangzhou, China) is a competitive receptor test in dipstick format for the rapid detection of residues of β-Lactams (penicillins and cephalosporins) and Tetracyclines in raw co-mingled cow's milk. The assay takes 6 minutes to complete.

The testing performed at MilkTestNZ is in line with the detection limits published by Ballya in the kit insert. Table 1 has the results of the testing performed at MilkTestNZ

MilkTestNZ has shown that the BT strip can detect the listed drugs at the concentrations indicated by the kit insert over a range of New Zealand milks. The over incubation of the reagents does not have an adverse effect on the strips performance.

Table 1: MilkTestNZ verified detection levels for Ballya BT Strip

	BallyA BT Strip Detection (µg/L)	NZ MRL (µg/L)	MilkTestNZ Verified Detection Limit (µg/L)
Ampicillin	4-6	4	6
Cefalonium	3-4	20	4
Cloxacillin	6-8	30	6
Penicillin G	1-1.5	2	1
Oxytetracycline	60-80	100	80
Sulfadiazine	ND	100	ND
Gentamicin	ND	100	ND
Tylosin	ND	50	ND

The verification testing has shown that he BT strip is suitable to use as a screening method for the detection of β-lactams and Tetracyclines in raw co-mingled milk.

Introduction

BT Sensors Test Kit (Ballya International Limited, Guangzhou, China) is a competitive receptor test in dipstick format for the rapid detection of residues of β-Lactams (penicillins and cephalosporins) and tetracyclines in raw co-mingled cow's milk. The assay takes six minutes to complete.

The detection capability of the kit was determined by Ballya International limited, the detection limits are printed in the kit instruction leaflet. Ballya international also contracted ILVO-T&V (Technology & Food Science Unit of the Institute for Agricultural and Fisheries Research of the Flemish Community) to perform a validation on the test kit.

This report is a verification that the test kit operates comparably with the two validations when performed on New Zealand milk in a New Zealand laboratory.

Background

The test is a competitive receptor test in dipstick format for the rapid detection of residues of β-Lactams (penicillins and cephalosporins) and tetracyclines in raw co-mingled cow's milk.

The test strip consists of three lines: A control line, a tetracycline receptor line and a β-lactam line.

Figure 1: Strip layout as shown in the BT Sensor kit pamphletFigure 1 shows that layout of the strip as displayed in the pamphlet that comes with the kit.

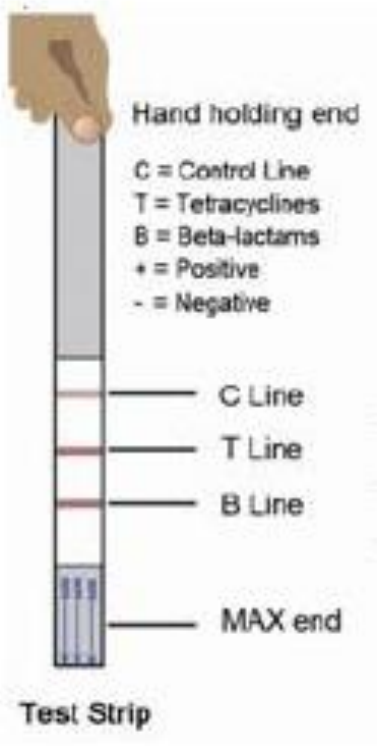

Figure 1: Strip layout as shown in the BT Sensor kit pamphlet

The strip is read by comparing the test lines to the control line. If the test line is lighter than the control line then it is considered positive for that family of antibiotics.

Test Procedure

Before starting the test, make sure that the temperature of the reagents and the milk are at room temperature.

Open the pack and remove the required number of test strips and micro wells. These should be used within one hour. The unused strips must be sealed and kept free of moisture and sunlight.

- Take the milk sample at room temperature, and add 200 µL to the microwell. Repeatedly aspirate up and down for five time to mix the sample with the reagents in the well completely.
- Wait for three minutes. After three minutes insert the test strip into the microwell with the "MAX" end fully immersed in the well.
- After incubation for three minutes remove the strip and read the results
- Testing was performed over two days.
- Only a single reader was able to be used in reading the strips, Serial number NB307 XTGU7nNj7

Regulatory Requirements

Tests used to detect contamination with inhibitory substances need to be approved by the Ministry for Primary Industries (MPI). MPI have indicated that a minimum of four of the six most common β-lactam antibiotics need to be verified as being detected at MRL in order for the kit to be acceptable for use.

The International Dairy Federation (IDF) is currently in the process of producing a document detailing how to carry out validation and/or verification of inhibitory substance test kits. This verification is based on the requirements outlined in the IDF provisional standard.

Verification and Validation

Verification and validation are independent procedures that are used together for checking that a product, service, or system meets requirements and specifications and that it fulfils its intended purpose.

Validation

The procedure applied to a newly (not existing before, significantly different from an existing method or existing but without an initial validation report) developed analytical method in the originator laboratory which demonstrates that the method is fit for purpose.

Verification

The procedure applied to a method which has been previously validated, for the same matrix. The verification procedure should allow the receptor laboratory to demonstrate that the method will work reliably in that laboratory with locally sourced milk and is fit for purpose.

As the Ballya β-Lactam and tetracycline rapid dipstick has been validated in Europe and China this report is a verification that the kit performs equivalently under New Zealand conditions.

Methods

For each involved family of veterinary drugs, at least one representative pharmacologically active substance (by preference the most difficult compound to be determined or the most relevant compound) will be tested. For a specific receptor assay test at least one a representative of the family that the test is specific should be tested. Representative compounds of the family of antibiotics that the strip is not sensitive too will be tested at 500x MRL to indicate no cross reactivity. MPI have indicated that at least four of the six most common β-lactams/cephalosporin's should be included in the verification testing.

The compounds to be included in this verification study are

- Penicillin - β-lactams
- Cloxacillin - β-lactams
- Cefalonium - Cephalosporin
- Ampicillin - β-lactams
- Oxytetracycline - Tetracycline
- Sulfamethazine - Sulfonamide
- Tylosin - Macrolide
- Gentamicin - Aminoglycoside

Table A1 of appendix A contains the BallyA international published detection limits for the test kit for various compounds.

Spiking protocol

For each of the drugs, a master solution was created. The indicated detection limit from published data was determined and these were the starting point for the dilution series. Final samples were created in raw milk that was determined to be negative for inhibitory substances.
Master mixes were made with primary and secondary solvents as indicated by Pharmacopeia. These are listed in Table 3:Primary and Secondary solvents used in the creation of master mixesTable 3

From each of the master mixes, a further dilution series was created to get to a concentration that could be inoculated directly into the milk sample. The subsequent dilutions were made up in a secondary solvent to avoid any issues with protein binding to the compound. Further dilutions were made using the secondary solvent until the dilution was added to the raw milk. All samples were made up to a final volume of 5 mLs. Calculations for spiking are presented in Appendix C Tables A3 to A7.

The following general principles were followed when deciding on the concentration of the secondary dilutions:

- The volume of the secondary dilution inoculated into the milk sample should not be more than 500 µL in 5 mL of raw milk or 10% of the total volume.
- The master mix should be diluted to a point to minimise milk sample dilution with the master mix.

A sample at the detection limit was created and in some cases a concentration less than the indicated detection limit and greater than the indicated detection limit was created and tested as well. From each of the master mixes, at least four different milk samples were inoculated at the appropriate level. Each of the samples was tested multiple times so that at each inoculation level there were at least 20 replicates over four different samples tested.

Milk Sample selection

Milk samples were randomly selected from samples that had been tested negative with the Copan Milk Test. Only samples containing at least 35 mL were used.

Table A2 in Appendix B – Composition results for samples used in the BT Strip verification has the composition data of the samples selected for use in the trial. The composition of the samples covered the normal range encountered in milk during this period of the season. Table 2 lists the minimum and maximum values obtained for the samples used in the analysis.

Master mixes were all created on the day of testing and further dilutions were determined by use of a MS-Excel spreadsheet to determine optimal dilutions to achieve the required final spiked samples.

Table 2: Basic statistics on negative milk samples used to create test samples

	Fat (% m/v)	*Protein (% m/v)*	*Lactose (% m/v)*	*Total Solids (% m/v)*	*Cell Count (per mL)*	*Freezing Point (°C)*
Average value	4.81	4.04	4.92	14.37	200400	-0.524
Standard Deviation	0.70	0.39	0.06	1.05	78430	0.003
Min	3.67	3.56	4.89	12.86	136000	-0.526
Max	5.35	4.44	5.04	15.21	332000	-0.518

Table 3:Primary and Secondary solvents used in the creation of master mixes

Drug	***Initial Solvent***	***Secondary Solvent***
Ampicillin Sodium Salt	RODI H_2O	RODI H_2O
Cloxacillin Sodium	RODI H_2O	RODI H_2O
Cefalonium	DMSO	RODI H_2O
Oxytetracycline dihydrate	0.1 N HCl	RODI H_2O
Sulfadiazine	0.025 N NaOH for first 30% then RODI H_2O	RODI H_2O
Gentamicin	RODI H_2O	RODI H_2O
Penicillin G	RODI H_2O	RODI H_2O
Tylosin	Methanol for first 30%, then RODI H_20	RODI H_2O

Note RODI H_2O = Reverse Osmosis Deionised water

BT Strip lot information

A single batch of BT strips was supplied for use in the verification testing.

- Lot BT20160.0.G01Z, Exp 2017.03.02

Samples were also tested on a CMT batch as a reference point. CMT is the current bacterial inhibition assay in use for routine testing in the laboratory, and as such of interest to compare drug sensitivities. Various CMT batches were used for this comparison, all of which had been approved for use in the laboratory through routine batch verification procedures.

Strip interpretation

A strip reader was supplied by Ballya to read the strips. All strips were read through this instrument which consists of a smart phone in a special reading case. The strip is placed in the indicated position and the phone camera used to take an image of the strip. The application on the phone then compares the control line to the test line and determines if it is darker or lighter and indicates a positive or negative result.

Results

The results of the verification testing are shown in Table 4. The visual interpretation of the results for each compound supports the electronic reading of the strips.

Table 4: Results of the verification testing carried out on Ballya BT Strips

	Ballya BT Strip Detection (µg/L)	*NZ MRL (µg/L)*	*MilkTest BT Strip Verified Detection Limit (µg/L)*
Ampicillin	4-6	4	6
Cephalonium	3-4	20	4
Cloxacillin	6-8	30	6
Penicillin G	1-1.5	2	1
Oxytetracycline	60-80	100	80
Sulfadiazine	ND	100	ND
Gentamicin	ND	100	ND
Tylosin	ND	50	ND

Note: ND = Not Detected

Supplied Controls

Positive and negative control samples supplied by Ballya International were also tested on the strip. The positive control was a lyophilised sample containing 1.5 ppb of Pen G and 80 ppb of Tetracycline. This was reconstituted with 0.5 mL of negative milk. The negative control was a lyophilised milk sample that was reconstituted with 0.5 mL of sterile water. Both controls were run on the BT strips and produced expected results.

Other testing

As part of the verification, extended incubation time of the strip was investigated. The initial 3 minutes incubation of the reagent without a strip was increased to 4 minutes with no adverse effect on the results.
The strips were removed as close to 3 minutes as was possible but it was observed that the reaction on the strips continued to occur, changing the detection level in some cases. After approximately 5 minutes, the reaction appears to stop and no further development of the strips occurs.

Conclusion

The ILVO-T&V validation indicated detection levels for the tested β-lactams at 1 ppb, with a detection limit of 5 ppb for the Oxytetracycline, MilkTestNZ testing does not match this level of detection.

The testing performed at MilkTestNZ is in line with the detection limits published by BallyA in the kit insert.

MilkTestNZ has shown that the BT strip can detect the listed drugs at the concentrations indicated by the kit insert over a range of New Zealand milks. The over incubation of the reagents does not have an adverse effect on the strips performance.

Of note is the fact that the reaction occurring on the strips appears to continue for a while after the 3 minute incubation. This may cause the sensitivity of the strip to be different if the strip is read outside the recommended window.

The BT strip is suitable to use as a screening method for the detection of β-lactams and tetracyclines in raw co-mingled milk from New Zealand.

Appendices

Appendix A – Ballya published detection limits of BT Strip

Table A1: Ballya published detection limits of BT Strip

β-Lactams	MRL (µg/L)	Indicated Detection limit (µg/L)	Cephalosporin	MRL (µg/L)	Indicated Detection limit (µg/L)
Penicillin G	4	1-1.5	Cefquinome	20	10-15
Ampicillin	4	4-6	Cefacetrile	125	40-50
Amoxicillin	4	4-5	Cefalonium	20	3-4
Oxacillin	30	6-8	Cefalotin	-	30-40
Cloxacillin	30	6-8	Cefoperazone	60	7-10
Dicloxacillin	30	6-8	Cephapirin	60	10-15
Nafcillin	30	15-20	Ceftiofur	100	90-100
Tetracyclines	**MRL (µg/L)**	**Indicated Detection limit (µg/L)**	**Tetracyclines**	**MRL (µg/L)**	**Indicated Detection limit (µg/L)**
Tetracycline	100	10-20	Doxycycline	100	60-80
Oxytetracycline	100	60-80	Chlortetracycline	100	10-20

Appendix B – Composition results for samples used in the BT Strip verification

Table A2: Composition results for samples used in the BT Strip verification

SAMPLE	FAT (% m/v)	PROTEIN (% m/v)	LACTOSE (% m/v)	TOTAL SOLIDS (% m/v)	SOMATIC CELL COUNT (PER mL)	FREEZING POINT (°C)
1	4.59	3.56	4.89	13.68	195000	-0.523
2	5.15	4.28	4.9	14.94	136000	-0.526
3	5.35	4.24	5.04	15.21	145000	-0.526
4	3.67	3.68	4.89	12.86	194000	-0.525
5	5.27	4.44	4.91	15.18	332000	-0.518

Appendix C – Calculations for creation of Master mixes and the creation of spiked samples.

Table A3: Calculations for the creation of master mixes

	Weight (g)	Final volume (mL)	Conc (%)	Master Mix Concentration g/mL	Master Mix Concentration mg/mL	Master Mix Concentration µg/mL	Master Mix Concentration µg/L
Ampicillin	0.0087	10	0.96	0.00084	0.8352	835.2	835200
Penicillin	0.0047	20	0.993	0.00023	0.233355	233.355	233355
Cloxacillin	0.0113	10	0.976	0.00110	1.10288	1102.88	1102880
Oxytetracycline	0.0208	20	0.994	0.00103	1.03376	1033.76	1033760
Cephalonium	0.0011	10	0.994	0.00011	0.10934	109.34	109340
Tylosin	0.0008	5	0.955	0.00015	0.1528	152.8	152800
Gentamicin	0.0035	5	0.93	0.00065	0.651	651	651000
Sulfamethazine	0.0059	20	100	0.0003	0.295	295	295000

Table A4: Calculations for creation of spiked samples for Ampicillin and Cloxacillin at indicated concentrations

	Ampicillin		Cloxacillin
Initial concentration	835200	Initial concentration	1102880
1 in 1000 dilution	835.2	1 in 1000 dilution	1102.88
Final Volume 5 mL	(µL)	Final Volume 5 mL	(µL)
Volume to add to get 1µg/L	6.0	Volume to add to get 1µg/L	4.5
Volume to add to get 4µg/L	23.9	Volume to add to get 6µg/L	27.2
Volume to add to get 6µg/L	35.9	Volume to add to get 8µg/L	36.3

Table A5 Calculations to create Cefalonium and Penicillin spiked samples

	Cefalonium		Penicillin G
Initial concentration	109340	Initial concentration	233355
1 in 100 dilution	1093.4	1 in 1000 dilution	233.355
Final Volume 5 mL	(µL)	Final Volume 5 mL	(µL)
Volume to add to get 1µg/L	4.6	Volume to add to get 1µg/L	21.4
Volume to add to get 6µg/L	18.3	Volume to add to get 1.5µg/L	32.1
Volume to add to get 8µg/L	27.4	Volume to add to get 2µg/L	42.9

Table A6: Calculation to create Oxytetracycline and Sulfamethazine spiked samples

	Oxytetracycline		Sulfamethazine
Initial concentration	1033760	Initial concentration	295000
1 in 100 dilution	1033.76	No Dilution	295000
Final Volume 5 mL	(µL)	Final Volume 5 mL	(µL)
Volume to add to get 5µg/L	24.2	Volume to add to get 12500µg/L	211.9
Volume to add to get 60µg/L	290.2		
Volume to add to get 80µg/L	386.9		

Table A7: Calculations to create Tylosin and Gentamicin spiked samples

	Tylosin		Gentamicin
Initial concentration	152800	Initial concentration	651000
No Dilution	152800	1 in 10 dilution	651000
Final Volume 5 mL	(µL)	Final Volume 5 mL	(µL)
Volume to add to get 25000µg/L	818.1	Volume to add to get 5000µg/L	384