VALIDATION REPORT

Validation Title: Comparison Study between IDF Disk Assay and Delvotest® SP-NT

Method: Direct Parallel Comparison

Project Log Number: 11 – 12

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1. EXECUTIVE SUMMARY

Delvotest® SP-NT is a ready to use commercial kit for detection of antibiotics in milk. This method is commonly used by the global dairy industry for product safety testing of raw and pasteurised liquid milk and has been validated for this purpose. An evaluation was conducted by AsureQuality to investigate method performance of the Delvotest® SP-NT for testing the presence of antibiotics in milk powder products; Non Fat Milk Powder (SMP) Whole Milk Powder (WMP), Whey Protein Concentrate (WPC), Whey Protein Isolate (WPI) Milk Protein Concentrate (MPC) Colostrum powder and Nutritional powder.

The IDF disk assay method according to IDF Standard 57: 1970 is routinely used in New Zealand for inhibitory substances detection in milk powder products and this technique was used in the study for comparison purposes.

A selection of antibiotics commonly used in New Zealand for animal husbandry were spiked into products at various concentrations, including the EU maximum residue limit (MRL). Four classes of antibiotics were represented including ß-lactams (Penicillin G, Ampicillin, Amoxicillin, Cloxacillin, Cephalonium), Tetracycline, Sulphathiazole and Streptomycin.

This study reports equivalent or improved detection sensitive at EU MRL limits for the ß-lactams compounds by the Delvotest® SP-NT method compared to the IDF disk assay technique. However, both methods were unable to detect the Sulphonimides, Tetracyclines or Streptomycin compounds included in this study at the EU MRL or 1.5 times the MRL in the presence of any dairy product matrices evaluated. The Delvotest® SP-NT was able to successfully detect all compounds at all concentrations in a blank reagent water matrix, whereas the IDF disk assay did not express the same sensitivity.

Being a commercially prepared kit in a ready to use format, the Delvotest® SP-NT Method was simple to perform and demonstrated exceptional repeatability through-out the study. The Delvotest® SP-NT method offers a reliable alternative to IDF disk assay method for the detection of inhibitory substances in milk powder products.

INTRODUCTION

This study has been designed in conjunction with Fonterra to compare the performance of Delvotest® SP-NT (DSM Food Specialties B.V. The Netherlands), with NZTM2 51.1 Inhibitory Substances for Milk and Milk Powders and NZTM2 51.2 Inhibitory Substances for Milk Protein Products, for the detection of inhibitory substances in various milk powder products. Both NZTM methods are based on IDF Standard 57: 1970. Detection of Penicillin in Milk by Disk Assay Technique.

The IDF disk assay method was first published in 1970 and then republished in 1986 and 1991 in subsequent IDF Bulletins. The method uses *Bacillus stearothermophilus* as the indicator test organism. The sample to be tested diffuses through an agar-based nutrient medium that has been uniformly seeded with spores of the susceptible test organism. The media is prepared in a large square assay plate (300mm x 300mm) and samples are placed on the solid agar surface using paper disks. Upon incubation, the indicator organism grows in the agar while a clear zone of inhibition around any of the disks, indicates the presence of inhibitor(s). The inhibition zone is measured and compared to corresponding zones on the same large plate from known concentration reference standards.

The Delvotest® SP-NT test kit also uses *Bacillus stearothermophilus* as the indicator organism; however the commercially prepared nutrient agar is seeded with spores and incorporates a dye, which serves as an indicator of growth. Each tube, containing nutrient agar, spores and dye, is inoculated with an individual sample. After incubation, spore germination and growth changes the colour of the media from purple to yellow. The absence of colour change in the media after a specified incubation time indicates the presence of an inhibitory substance(s).

The disk assay method is used by the New Zealand dairy industry to test milk powder products for inhibitory substances and is currently the only method approved by the New Zealand Food Safety Authority (NZFSA/MAF) for domestic and export food safety testing. Delvo SP NT is used worldwide by multinational dairy companies for inhibitory substances detection in milk powder products, however DSM have not formally validated the method for products other than liquid milk.

Screening assays for antibiotic residues in milk are efficient monitoring procedures in that they are simple and rapid, permitting numerous samples to be screened. However, they are non-specific and only respond to biologically active residues that inhibit the growth of the

indicator organism. This can be both an advantage and a disadvantage as some naturally occurring substances in milk and milk products can lead to zone formation.

2. OBJECTIVE

This study was conducted to evaluate the ability of both methods to detect various antibiotics in common milk powder products. The antibiotics selected are those commonly used in animal husbandry in New Zealand and represent a range of compounds including ß-lactams, tetracyclines, sulphonamides and aminoglyocides.

3. REFERENCES

- Delvotest® SP-NT product insert (DSM Food Specialties B.V. The Netherlands).
- IDF Standard 57: 1970. Detection of Penicillin in Milk by a Disk Assay Technique using the test modified as described in NZTM2 51.1 for milk powders and NZTM2 51.2 for milk proteins.

4. VALIDATION PARAMETERS

4.1 Sample Matrices

The sample types used in the study are:

- Non Fat Milk Powder (SMP)
- Whole Milk Powder (WMP)
- Whey Protein Concentrate (WPC)
- Whey Protein Isolate (WPI)
- Milk Protein Concentrate (MPC)
- Colostrum powder
- Nutritional powder

4.2 Sample Preparation

Each sample type was reconstituted 1:10 according to NZTM2 sample preparation guidelines.

4.3 Antibiotics

Eight antibiotics were evaluated at; EU MRL levels, and two other levels approximately at the detection limit for either test. Levels are shown in the table 1 below.

Table 1. Antibiotic types and concentrations used for matrix spiking.

Class	Antibiotic	EU MRL (ppb)	Other 1 (ppb)	Other 2 (ppb)
ß-lactam	Penicillin G	4	3	2
ß-lactam	Ampicillin	4	3	2
ß-lactam	Amoxicillin	4	9	6
ß-lactam	Cloxacillin	30	20	10
ß-lactam	Cephalonium	10	7.5	5
Tetracycline	Tetracycline	100	75	200
Sulphonamide	Sulphathiazole	200*	375	250
Aminoglycoside	Streptomycin	200	450	300

Note: *ppb is equivalent to µg/Kg*

*double the EU MRL Level of 100ppb, but chosen because this is the detection limit of the test.

1. Pharmacopeia grade antibiotics material accompanied by certificates of analysis according USP standards, or equivalent, were utilised in the trial.
2. The reported purity of each raw material was taken into account when calculating the concentration of each standard preparation.
3. Working solutions for each antibiotic were prepared using RO Water free of inhibitory substances. Appropriate volumes of the working solution were added to 50 mL of the reconstituted sample to achieve the final target concentration of the antibiotic. Effective mixing was performed.
4. All solutions were prepared using volumetric glassware. Testing commenced within 1 hour of sample spiking.

4.4 Trial Procedure

1. Each sample matrix was spiked to achieve an antibiotic concentration equal to; the MRL level, and two other levels approximately at the detection limit for either test.
2. Each sample was tested in parallel by both IDF Disk Assay (according to NZTM2 51.1 or 51.2) and Delvotest® SP-NT. Each sample was tested in duplicate on five large assay plates for the IDF disk assay method and 10 replicates for the Delvotest® SP-NT test.

   The test protocol involved evaluation of 1,470 samples tested by both Delvotest® SP-NT and IDF disk assay in addition to the quality control samples listed in 4.5.

4.5 Quality Control

Positive control

Each concentration of antibiotic was prepared in deionised water as a matrix blank and tested as the positive control. Ten replicates of each concentration were tested by the IDF method and 5 replicates were tested by the Delvotest® SP-NT method.

Blank control

Each sample matrix type was tested ten times without added antibiotic to represent test blank samples.

5. RESULTS AND OBSERVATIONS

Table 2: Average zone size, in mm, expressed for each antibiotic/matrix combination using IDF Disk Assay method.

Antibiotic	Concentration (ppb)	IDF Method (Average Zone Size in mm)							
		Skim Milk Powder	Whole Milk Powder	Whey protein Conc.	Whey Protein Isolate	Milk Protein Conc.	Colostrum Powder	Nutritional Powder	Deionised water
Penicillin G	2	13.1	13.1	13.1	13.3	13.2	13.4	13.1	13.1
	3	15.1	14.7	14.7	14.9	14.7	15.2	14.9	14.8
	4	16.1	15.9	15.8	16.1	16.0	16.3	16.1	16.1
Ampicillin	2	13.0	13.0	13.1	13.1	13.1	13.3	13.0	13.0
	3	13.2	13.2	13.4	13.4	13.4	13.8	13.3	13.2
	4	13.6	13.8	13.8	13.7	13.7	14.1	13.7	13.8
Amoxicillin	4	13.4	13.4	13.4	13.3	13.4	13.4	13.4	13.4
	6	14.6	14.6	14.7	14.7	14.7	14.7	14.8	14.7
	9	15.7	15.8	15.7	16.0	16.0	15.9	15.8	15.9
Cloxacillin	10	No Zone	No Zone	No Zone	No Zone	No Zone	13.2	No Zone	No Zone
	20	13.1	13.2	13.2	13.3	13.1	13.2	13.2	13.2
	30	13.5	13.7	13.7	13.8	13.6	13.9	13.9	14.1
Cephalonium	5	No Zone	No Zone	No Zone	No Zone	No Zone	13.3	No Zone	No Zone
	7.5	No Zone	No Zone	No Zone	No Zone	No Zone	13.3	No Zone	No Zone
	10	No Zone	No Zone	No Zone	No Zone	No Zone	13.4	No Zone	No Zone
Tetracycline	75	No Zone	No Zone	No Zone	No Zone	No Zone	13.3	No Zone	No Zone
	100	No Zone	No Zone	No Zone	No Zone	No Zone	13.3	No Zone	No Zone
	200	No Zone	No Zone	No Zone	No Zone	No Zone	13.4	No Zone	13.1
Sulphathiazole	200	No Zone	No Zone	No Zone	No Zone	No Zone	13.3	No Zone	No Zone
	250	No Zone	No Zone	No Zone	No Zone	No Zone	13.3	No Zone	No Zone
	375	No Zone	No Zone	No Zone	No Zone	No Zone	13.4	No Zone	No Zone
Streptomycin	200	No Zone	No Zone	No Zone	No Zone	No Zone	13.3	No Zone	No Zone
	300	No Zone	No Zone	No Zone	No Zone	No Zone	13.3	No Zone	No Zone
	450	No Zone	No Zone	No Zone	No Zone	No Zone	13.4	No Zone	No Zone

Table 3: Test outcome expression for each antibiotic/matrix combination using Delvotest® SP-NT.

Antibiotic	Concentration (ppb)	Delvotest® SP-NT Method							
		Skim Milk Powder	Whole Milk Powder	Whey protein Conc.	Whey Protein Isolate	Milk Protein Conc.	Colostrum Powder	Nutritional Powder	Deionised water
Penicillin G	2	Pos (DL)	Pos	Pos	Pos (DL)	Pos (DL)	Pos (DL)	Pos (DL)	Pos
	3	Pos	Pos	Pos	Pos	Pos	Pos	Pos	Pos
	4	Pos	Pos	Pos	Pos	Pos	Pos	Pos	Pos
Ampicillin	2	Neg	Indeterminate	Neg	Neg	Neg	Neg	Indeterminate	Pos
	3	Pos	Pos	Pos	Pos	Pos	Pos	Pos	Pos
	4	Pos	Pos	Pos	Pos	Pos	Pos	Pos	Pos
Amoxicillin	4	Pos (DL)	Pos (DL)	Pos (DL)	Pos (DL)	Pos (DL)	Pos (DL)	Pos (DL)	Pos (DL)
	6	Pos	Pos	Pos	Pos	Pos	Pos	Pos	Pos
	9	Pos	Pos	Pos	Pos	Pos	Pos	Pos	Pos
Cloxacillin	10	Neg	Neg	Neg	Neg	Neg	Neg	Neg	Pos (DL)
	20	Pos (DL)	Pos (DL)	Pos (DL)	Pos (DL)	Pos (DL)	Neg	Pos (DL)	Pos
	30	Pos	Pos	Pos	Pos	Pos	Pos	Pos	Pos
Cephalonium	5	Neg	Neg	Neg	Neg	Neg	Neg	Neg	Pos (DL)
	7.5	Neg	Neg	Neg	Neg	Neg	Neg	Neg	Pos
	10	Pos (DL)	Pos (DL)	Pos (DL)	Pos (DL)	Pos (DL)	Pos (DL)	Pos (DL)	Pos
Tetracycline	75	Neg	Neg	Neg	Neg	Neg	Neg	Neg	Pos (DL)
	100	Neg	Neg	Neg	Neg	Neg	Neg	Neg	Pos
	200	Neg	Neg	Neg	Neg	Neg	Neg	Neg	Pos
Sulphathiazole	200	Neg	Neg	Neg	Neg	Neg	Neg	Neg	Neg
	250	Neg	Neg	Neg	Neg	Neg	Neg	Neg	Neg
	375	Neg	Neg	Neg	Neg	Neg	Neg	Neg	Neg
Streptomycin	200	Neg	Neg	Neg	Neg	Neg	Neg	Neg	Pos (DL)
	300	Neg	Neg	Neg	Neg	Neg	Neg	Neg	Pos
	450	Neg	Neg	Neg	Neg	Neg	Neg	Pos (DL)	Pos

Key:

Neg	Negative (complete colour change yellow)
Pos (DL)	Positive (partial colour change purple/yellow)
Pos	Positive (No colour change - purple)

Table 4: Summary of detection limits for each antibiotic and matrix type.

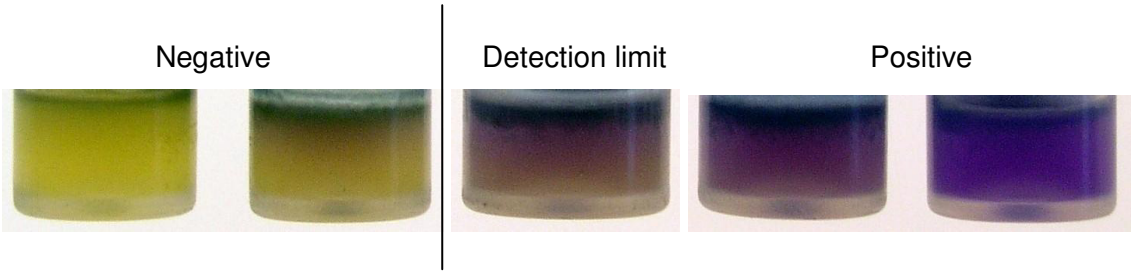
Antibiotic	Test Method	Detection Limit expressed as ppb (*µg/kg*)							
		Skim Milk Powder	**Whole Milk Powder**	**Whey protein Conc.**	**Whey Protein Isolate**	**Milk Protein Conc.**	**Colostrum Powder**	**Nutritional Powder**	**Deionised water**
Penicillin G (EU MRL 4 *µg/kg)*	**Delvotest® SP-NT**	2	2	2	2	2	2	2	2
	IDF Disk Assay	2	2	2	2	2	2	2	2
Ampicillin (EU MRL 4 *µg/kg)*	**Delvotest® SP-NT**	3	2	3	3	3	3	2	2
	IDF Disk Assay	2	2	2	2	2	2	2	2
Amoxicillin (EU MRL 4 *µg/kg)*	**Delvotest® SP-NT**	4	4	4	4	4	4	4	4
	IDF Disk Assay	4	4	4	4	4	4	4	4
Cloxacillin (EU MRL 30 *µg/kg)*	**Delvotest® SP-NT**	20	20	20	20	20	30	20	10
	IDF Disk Assay	20	20	20	20	20	20	20	20
Cephalonium (EU MRL 20 *µg/kg)*	**Delvotest® SP-NT**	10	10	10	10	10	N/A	10	5
	IDF Disk Assay	>10	>10	>10	>10	>10	N/A	>10	>10
Tetracycline (EU MRL 100 *µg/kg)*	**Delvotest® SP-NT**	>200	>200	>200	>200	>200	N/A	>200	75
	IDF Disk Assay	>200	>200	>200	>200	>200	N/A	>200	200
Sulphathiazole (EU MRL 100 *µg/kg)*	**Delvotest® SP-NT**	>375	>375	>375	>375	>375	N/A	>375	>375
	IDF Disk Assay	>375	>375	>375	>375	>375	N/A	>375	>375
Streptomycin (EU MRL 200 *µg/kg)*	**Delvotest® SP-NT**	>450	>450	>450	>450	>450	N/A	450	200
	IDF Disk Assay	>450	>450	>450	>450	>450	N/A	>450	>450

6. ACCEPTANCE CRITERIA

For the Delvotest, the results were read after 3 hours of incubation and visually assessed for colour change. The colour change scale detailed in the Delvotest® SP-NT product insert was used as the interpretation guide. See below figure 1.

For the IDF disk assay the zone size was reported as the average of the duplicate disks in millimetres (mm).

Figure 1: Delvotest® reading colour chart interpretation



The study outcome will be considered acceptable if the Delvotest® SP-NT test consistently yields a positive result at or below the MRL for each antibiotic in the trial. However, an outcome where the Delvotest® SP-NT shows equivalent or better sensitivity than the NZTM methods for non ß-lactam antibiotics at higher than MRL levels is also likely to be acceptable.

6. INTERPRETATION AND CONCLUSION

Based on the results obtained from this study it can be concluded the Delvotest® SP-NT method provides an equivalent or improved method for the detection of ß-lactam antibiotics at EU MRL concentrations in the milk powder products evaluated, compared to the disk assay method according to IDF. For Tetracycline, Sulphathiazole and Streptomycin the Delvotest® SP-NT method also expressed an equivalent or improved detection sensitivity, albeit, some antibiotic/matrix combinations were not detected by either method.

The presence of any zone size in the IDF method constitutes a positive result; however this is not in the case for the NZTM2 method where a zone size of 1 mm from the disk edge is stipulated as a minimum result. However, and even using the IDF interpretation, the sensitivity for the disk method could not be reproduced in this study. And despite the Delvotest® SP-NT method being able to detected lower concentrations of Cephalonium, Tetracycline and Streptomycin compared to the IDF method when in deionised water, the presence of milk powder product was shown to affect the kit performance.

Neither test method was able to detect Sulphathiazole at the concentrations used in this study.

For the Delvotest® SP-NT the detection limit for Cloxacillin in colostrum was higher at 30 ppb than for the other dairy products where this antibiotic was detected at 20 ppb. All other antibiotics showed a similar sensitivity regardless of dairy product matrix for both the Delvotest® SP-NT and IDF disk assay.

All sample types used in the study were evaluated for interference by testing each sample type after reconstitution. No adverse matrix effects were noted for the Delvotest® SP-NT test i.e. all samples expressed colour change at 3 hours. In contrast, the colostrum product consistently expressed a positive zone of inhibition in the IDF test through-out the study. The zone of inhibition size was between 13.2 mm – 13.6 mm and was considered a baseline effect for the purposes of this study. This observation provides some anecdotal evidence to suggest the Delvotest® SP-NT test may not be as prone to false positive responses as the IDF Disk assay test when the sample includes natural inhibitors such as immunoglobulins, lysosomes or other natural defense agents.

The length of incubation required to achieve a definitive outcome was dependant on the incubation method used. In a pilot study conducted prior to the commencement of the study, both air incubator (with an active air distribution fan) and a temperature controlled water bath were investigated as possible equipment to use for the study. We found incubation in a water bath produced a definitive colour change of a blank Delvotest® SP-NT kit in 3 hours. An additional 15-20 minutes was required when using an air incubator. Based on the pilot evaluation outcome, a water bath was used for incubation of the Delvotest® SP-NT kit in the actual study. For the IDF disk assay method an air incubator was used as per routine practise.

The repeatability of zone sizes in the IDF test for the same sample varied by up to 0.5mm at the highest concentration of antibiotics, which could lead to variation in the estimate of relative concentration when applying a standard curve interpolation. To a degree the variation is managed by plating duplicate disks in opposing locations on the large assay plate (using quasi Latin square positioning), however no quality control criteria is currently stated in the method to identify and interpret significant differences between duplicate zones. The current requirement to average estimates could lead to “damping” of result accuracy. In the disk assay method there are several factors that affect the size and appearance of zones of inhibition. The number of viable organisms used to inoculate the medium is critical because

the density of growth (and therefore visualization of the zones) is dependent on the initial numbers of organisms. The temperature of incubation also must be rapidly controlled because both the rate of growth of organism and rate of diffusion of inhibitor are a temperature related phenomena. Porosity of the medium also influences the rate of diffusion. In general, lower proportions of agar result in larger zones of inhibition. Other factors include the depth of agar, age of inoculum, technique for adding sample to the disk, and the sample matrix composition. In contrast, the Delvotest® SP-NT test is less vulnerable to technique variation and the superior repeatability was obvious in the study.