Validation of Alternative Isolation on Confirmation of *Cronobacter* Species in Powdered Infant Nutritionals, Milk Powders and Environmental Samples Following the Assurance® GDS for *Cronobacter* Tq II Assay

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Introduction

Cronobacter species is a pathogen of concern in infant nutritionals. An extension for a new rapid confirmation method for Assurance® GDS for *Cronobacter* Tq II was performed (Campden BRI, Expert Lab) in the food category of infant formula and infant cereals, including milk powder. This configuration was established by MicroVal, where an inclusivity and exclusivity study and a reduced sensitivity study was used to assess a new confirmation method to an existing qualitative method.

Purpose

To validate the next-day rapid confirmation of *Cronobacter* in infant nutritionals, milk powder, and environmental samples compared to the ISO reference method.

Methods

Follow MicroVal interpretation guidelines for a new confirmation method from a previously validated qualitative method:

- inclusivity and exclusivity study (100 strains)
- limited number of sensitivity samples

The study included 46 samples for the sensitivity analysis. Lyophilized cultures of *Cronobacter* were inoculated into foods and stabilized at room temperature for a minimum of 2 weeks. Samples (375 g) were enriched 1:10 in the appropriate media for 24 h and rapidly confirmed by either direct streak or immunomagnetic separation (IMS) onto 3 chromogenic plate agars (2 agars for IMS).
All inclusivity and exclusivity isolates were streaked onto all chromogenic plate types, followed by MALDI ToF and biochemical analysis of typical colonies.

Results

Study findings were obtained through both direct streak and IMS methodologies employed in the isolation and confirmation of *Cronobacter*. These results demonstrate favorable concordance of the sensitivity study, encompassing 46 tested samples. All results fell below the stipulated Acceptability Limit (AL).

Fig 1. Workflow – alternative method

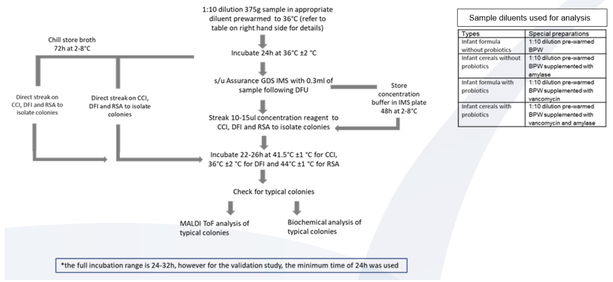

Table 1. Inclusivity Results

Presumptive results

Number of cultures positive	Number positive results		
	CCI	DFI	RSA
	97/100	99/100	100/100

CCI
3 discrepant isolates (CRA 5181, 5196 and 5197) were confirmed by MALDI ToF to be Cronobacter spp.

DFI
1 discrepant isolate (CRA 17639, CCUG 28863) were confirmed by MALDI ToF to be Cronobacter spp.

Protocol	ND-PD	AL	Evaluation	ND-PD	AL	Evaluation
Direct streak from the initial enrichment	0	3	Accepted	0	3	Accepted

The AL for the inclusivity study were met for the 100 isolates analysed

The calculated ND-PD and ND+PD were lower than the AL required for 100 inclusivity isolates

Table 2. Exclusivity Results

Presumptive results

Number of isolates giving expected results		
CCI	DFI	RSA
99/100	98/100	99/100

Identification of presumptive positive colonies

Code	Organism	Agars positive	Confirmation
47	*Franconibacter helveticus* CRA 17678	CCI, DFI, RSA	*Franconibacter helveticus*
52	*Klebsiella oxytoca* CRA 15926	DFI	*Klebsiella oxytoca*

Protocol	ND-PD	AL	Evaluation	ND-PD	AL	Evaluation
Direct streak from the initial enrichment	0	2	Accepted	0	2	Accepted

The AL for the exclusivity study were met. The calculated ND-PD and ND+PD were lower than the AL required for 100 exclusivity isolates

Table 3: Alternative confirmation: Direct and IMS

Sample type	Direct streak enrichment at time of GDS analysis			Direct streak enrichment following storage at 2-8°C for 72h			Plate resuspension IMS at time of GDS analysis			Plate resuspension IMS following storage at 2-8°C for 48h		
	CCI	DFI*	RSA*	CCI*	DFI*	RSA*	CCI	DFI	RSA*	CCI	DFI	RSA*
Infant Formula without Probiotics	✓	✓	✓	✓	✓	✓	✓	✓		✓	✓	
Infant Cereals without Probiotics	✓	✓	✓	✓	✓	✓	✓	✓		✓	✓	
Non-probiotic Ingredients (except dry milk)	✓	✓	✓	✓	✓	✓	✓	✓		✓	✓	
Infant Formula with Probiotics	✓	✓	✓	✓	✓	✓	✓	✓		✓	✓	
Infant Cereals with Probiotics	✓	✓	✓	✓	✓	✓	✓	✓		✓	✓	
Dry milk (including NFDM)	✓						✓	✓		✓	✓	

Table 4-5: Sensitivity – Alt Confirmation (Direct)

Direct streaks from the initial sample enrichment

Category	Food type	No samples	No. of colonies confirmed for each agar and confirmation method combination						Number of biochemical confirmations per food type
			CCI		DFI		RSA		
			Maldi	API	Maldi	API	Maldi	API	
Infant formula and cereals	Infant formula without probiotics	5	5	5	5	-	5	-	15
	Infant cereals without probiotics	5	5	-	5	5	5	-	
	Non-probiotic ingredients (exc. dry milk)	5	5	-	5	-	5	5	
	Total	15	15	5	15	5	15	5	
	Infant formula with probiotics	8	8	4	8	4	8	-	16
	Infant cereals with probiotics	8	8	4	8	-	8	4	
	Total	16	16	8	16	4	16	4	
	Dry milk	15	15	5	np	np	np	np	5
	Total	15	15	5	15	5	15	5	
	Overall total	46	46	18	31	9	31	9	36

Summary of Results

Category	Food type	No of positive confirmed samples for each agar and confirmation method combination					
		CCI		DFI		RSA	
		Maldi	Biochemical	Maldi	Biochemical	Maldi	Biochemical
Infant formula and cereals	Infant formula without probiotics	5/5	5/5	5/5	nt	5/5	nt
	Infant cereals without probiotics	5/5	nt	5/5	5/5	5/5	nt
	Non-probiotic ingredients (exc. dry milk)	5/5	nt	5/5	nt	5/5	5/5
	Total	15	5	15	5	15	5
	Infant formula with probiotics	8/8	4/4	8/8	4/4	8/8	nt
	Infant cereals with probiotics	7/8*	3/4*	8/8	nt	8/8	4/4
	Total	14	7	16	4	16	4
	Dry milk	15/15	5/5	np	np	np	np
	Total	15	5	0	0	0	0
	Overall total	45/46	17/18	31/31	9/9	31/31	9/9

Summary:

Direct streak protocol gave good agreement with the expected results for the 46 samples tested in the sensitivity study for all three plate types (CCI, DFI, RSA)

Tables 6-7: Sensitivity – Alt Confirmation (IMS)

Streaks from the IMS concentration reagent

Category	Food type	No samples	No. of colonies confirmed for each agar and confirmation method combination						Number of biochemical confirmations per food type
			CCI		DFI		RSA		
			Maldi	API	Maldi	API	Maldi	API	
Infant formula and cereals	Infant formula without probiotics	5	5	5	5	-*	np	np	10
	Infant cereals without probiotics	5	5	-	5	5	np	np	
	Non-probiotic ingredients (exc. dry milk)	5	5	-	5	-	np	np	
	Total	15	15	5	15	5	0	0	
	Infant formula with probiotics	8	8	4	8	4	np	np	12
	Infant cereals with probiotics	8	8	4	8	-	np	np	
	Total	16	16	8	16	4	0	0	
	Dry milk	15	15	5	15	5	np	np	10
	Total	15	15	5	15	5	0	0	
	Overall total	46	46	18	46	14	0	0	32

Summary of Results

Category	Food type	No of positive confirmed samples for each agar and confirmation method combination					
		CCI		DFI		RSA	
		Maldi	Biochemical	Maldi	Biochemical	Maldi	Biochemical
Infant formula and cereals	Infant formula without probiotics	5/5	5/5	5/5	nt	np	np
	Infant cereals without probiotics	5/5	nt	5/5	5/5	np	np
	Non-probiotic ingredients (exc. dry milk)	4/5*	nt	4/5*	nt	np	np
	Total	14	5	14	5	np	np
	Infant formula with probiotics	8/8	4/4	8/8	4/4	np	np
	Infant cereals with probiotics	7/8*	4/4	8/8	-	np	np
	Total	15	8	16	4	np	np
	Dry milk	13/15*	5/5	15	5/5	np	np
	Total	13	5	15	5	np	np
	Overall total	42/46	18/18	45/46	14/14	np	np

Summary:

Resuspension plate protocol gave good agreement with the expected results for the 46 samples tested for all two plate types (CCI, DFI).

*No presumptive positive colonies seen from samples with initial result of no detection.

Discussion

This new alternative isolation and confirmation method also compared different chromogenic agars for *Cronobacter*. During development, it was noted that RSA experienced difficulty in the recovery of *Cronobacter* when plated from immunomagnetic beads compared to the other plate types. It is unknown the cause of this discrepancy.

Similarly, isolation by direct streaking from NFDM enrichments was only accomplished onto CCI agar at time of enrichment. It is speculated that the acidic environment is the cause of this injury to the Cronobacter that prevents isolation by direct streak method. Isolation by IMS methods from NFDM enrichments were not affected, either at time of enrichment of after storage of the resuspension plate. Cronobacter were removed from acidic conditions by the IMS procedure.

Conclusion

This method comparison study gathers data for a new alternative isolation and confirmation method. The study was performed according to the MicroVal interpretation guidelines for ISO 16140-Part 6 (2019) as an extension study for a new confirmation method from a previously validated qualitative method.

These two new methods provide rapid isolation and confirmation for *Cronobacter* and are selective and specific following Assurance® GDS for *Cronobacter* Tq II detection method.

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