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Method Validation Report for *Enterobacteriaceae* as per ISO 21528-2:2017

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ABSTRACT

The family *Enterobacteriaceae* consists of a heterogeneous group of rod-shaped, gram-negative bacteria, varying in their ecological niche, host range, and pathogenic potential for humans, animals, insects, and plants. The method for the enumeration of *Enterobacteriaceae* in food samples as per ISO 21528-2:2017, was validated for a project involving the use of the standard test method in commercial laboratories across different regions. The study was performed to attend precision in terms of linearity, repeatability, reproducibility, accuracy, and limit of detection. Sampled were artificially contaminated (spiked) to achieve desired concentration of bacterial cells for validating the method. The precision depended on the type of the samples being analysed.

Keywords- *Enterobacteriaceae*, Method Validation, Linearity, Spike, *Escherichia coli*.

INTRODUCTION

One serious threat to public health in both developed and developing countries is the microbial contamination of food. This problem poses a great challenge and consequently has economic implications. Causes of microbial contamination are diverse and these maybe natural, environmental or technological.

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The microbiological quality of most ready to- eat foods is of great significance to human health because they require minimal or no processing when consumed. [1, 6]

Today, many methods exist that are used to assess the microbiological safety and quality of raw materials and finished food products and to monitor the microbiological status of manufacturing processes. The developers, end-users and public health authorities need a reliable common protocol for the validation of such alternative methods. [2, 3, 5, 7]

A test method must be shown to be fit for purpose so that a facility's customers can have confidence in the results produced by its application. Method validation provides objective evidence that a method is fit for purpose, meaning that the requirements for a specific intended use are fulfilled.

Validation is always a balance between costs, risks, and technical possibilities. The extent of validation required will depend on the status of the method under consideration and the needs relating to its intended application. [2, 3, 7]

The family *Enterobacteriaceae* consists of a heterogeneous group of rod-shaped, gram-negative bacteria, varying in their ecological niche, host range, and pathogenic potential for humans, animals,
insects, and plants. Members of the family are aerobes or facultative anaerobes, are motile by peritrichous flagella or nonmotile, do not form endospores or microcysts, and are chemoorganotrophic
with both respiratory and fermentative metabolisms, and most grow well at 22–35 ^{o}C. Foodborne pathogens in the family
Enterobacteriaceae generally do not demonstrate an atypical heat resistance, and they are inactivated at temperatures lower than the pasteurization temperature for milk (72 ^{o}C for 15s). The family *Enterobacteriaceae* contains more than 50 genera and many species, and there are several human pathogens within this family. [1, 6, 7]

The ISO 21528-2:2017 International Standard describes a horizontal method for the enumeration of viable *Enterobacteriaceae*. This test method is applicable to products intended for human consumption and the feeding of animals, and environmental samples in food production and food handling. [7]

For the validation the method was challenged with two different food matrices. For this study raw chicken breast and Milk sample were used. In addition, a reference material was used to identify any serious errors in any of the participant's performance and to determine the maximum precision possible as the ISO 21528-2:2017 is quantitative method. The food samples were artificially inoculated to achieve desired inoculum levels and homogeneity. These materials were tested extensively by four different trained analyst's and the data from the testing was used to calculate the following in relation with the food type and inoculum level -

1. Linearity- which is the property of a mathematical relationship or function that can be graphically represented as a straight line. Therefore, linearity uncertainty would the uncertainty associated with non-linear behaviour observed across the range of an assumed linear function.
2. Repeatability is defined as the ability of the analytical procedure to obtain test results within a given range of accuracy and precision when the same sample is repeated in accordance with the same method protocol. Chicken breast and Milk samples were spiked with known concentration of culture suspension and the same sample was repeated 10 times, to calculate the Mean, SD and %RSD.
3. Reproducibility is defined as the ability of the analytical procedure to obtain test results within a given range of accuracy and precision when the same sample is performed by different analyst in accordance with the same method protocol. Chicken Breast and Milk samples were spiked with known concentration of bacterial cell suspension and the same sample was repeated by four different analysts', to calculate the Mean, SD and %RSD.

Accuracy is the closeness of agreement between a test result and accepted reference value. Accuracy of the tested sample was determined by using the following formulae –

(Test Result/Expected Value) *100

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EXPERIMENTAL METHODS

Preparation of Stock Culture.

1 lyophilized pellet of *Escherichia coli* ATCC 8739 cultures provided by Microbiologic' s is added to sterile phosphate buffer saline which was pre-incubated at 35ºC for 30 minutes. This suspension is incubated at 35ºC for 24 hrs under anaerobic conditions. Prepare a serial dilution of the culture to verify the known concentration.

Spiking of samples.

Sterile chicken breast and sterile milk samples were spiked with the cell suspension which was prepared in 2.1.

The samples were spiked with different concentration of cell suspension ranging from 10^{-5} to 10^{-7} CFU/ml.

Method Validation

To perform the method validation different parameters were conducted which were-Linearity, Repeatability, Reproducibility, Accuracy and Limit of Detection.

RESULTS

Stock Culture

Table 1- Stock Culture- *Escherichia coli* ATCC 10536

Dilution	Sterile Distilled Water										Mean	SD	% RSD
10^{-5}	133	130	136	137	136	139	134	135	131	136	134.7	2.75	0.20
10^{-6}	17	11	10	12	13	15	12	11	10	9	120.0	2.44	2.04
10^{-7}	1	1	0	0	1	1	1	2	0	0	7.0	0.67	9.64
10^{-8}	0	0	0	0	0	0	0	0	0	0	0	0	0

Linearity

Table 2 – Linearity

Samples / Dilution	Milk					Chicken Breast				
	Values		Mean	SD	% RSD	Values		Mean	SD	% RSD
10^{-5}	120	124	122	2.82	2.31	125	127	126	1.41	1.12
10^{-6}	11	10	10.5	0.70	6.73	12	13	12.5	0.70	5.65
10^{-7}	0	0	0	0	0	0	0	0	0	0

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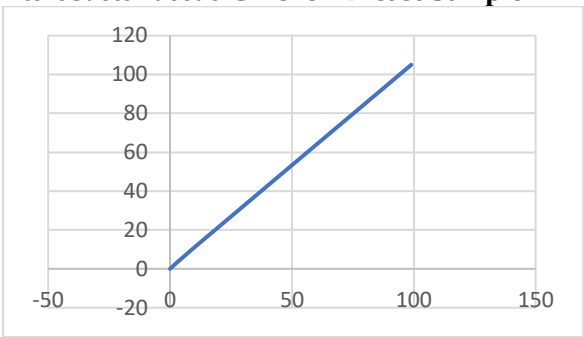
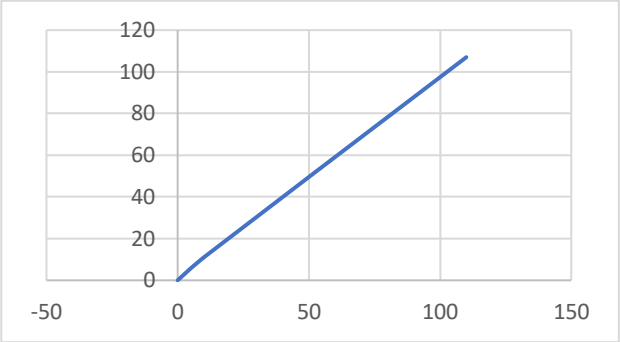
Graph 1a – Linearity – *Enterobacteriaceae* Chicken Breast Sample



Graph 1b - Linearity – *Enterobacteriaceae* Milk Sample



Repeatability

The results for the analysis are demonstrated in the tables which are given below.

Table 3a – Repeatability for Chicken Breast Analyst 1

Dilution	Repeatability										Mean	SD	% RSD
	1	2	3	4	5	6	7	8	9	10			
10^{-5}	130	125	128	131	134	129	134	126	128	129	129.4	2.98	2.30
10^{-6}	11	10	9	13	12	10	10	11	12	13	11.1	1.37	12.34

Table 3b – Repeatability for Chicken Breast Analyst 2

Dilution	Repeatability										Mean	SD	% RSD
	1	2	3	4	5	6	7	8	9	10			
10^{-5}	127	126	130	134	133	126	125	129	126	128	128.4	3.09	2.41
10^{-6}	12	12	10	13	9	10	11	12	10	9	10.8	1.39	12.94

Table 3c – Repeatability for Chicken Breast Analyst 3

Dilution	Repeatability										Mean	SD	% RSD
	1	2	3	4	5	6	7	8	9	10			
10^{-5}	125	127	131	130	134	131	125	128	125	129	128.5	3.06	2.38
10^{-6}	12	12	10	13	9	10	11	12	10	9	10.8	1.39	12.94

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Table 3d – Repeatability for Chicken Breast Analyst 4

Dilution	Repeatability										Mean	SD	% RSD
	1	2	3	4	5	6	7	8	9	10			
10^{-5}	132	128	129	134	124	126	129	130	126	127	128.5	2.99	2.32
10^{-6}	12	12	10	13	9	10	11	12	10	9	10.8	1.39	12.94

Table 3a – Repeatability for Milk Analyst 1

Dilution	Repeatability										Mean	SD	% RSD
	1	2	3	4	5	6	7	8	9	10			
10^{-5}	125	120	119	129	124	121	127	124	120	129	123.8	3.74	3.02
10^{-6}	10	9	11	10	9	10	11	12	9	12	10.3	1.16	11.26

Table 3b – Repeatability for Milk Analyst 2

Dilution	Repeatability										Mean	SD	% RSD
	1	2	3	4	5	6	7	8	9	10			
10^{-5}	119	122	124	126	124	120	121	126	121	128	123.1	2.96	2.40
10^{-6}	11	11	9	11	12	9	12	9	9	12	10.5	1.35	12.89

Table 3c – Repeatability for Milk Analyst 3

Dilution	Repeatability										Mean	SD	% RSD
	1	2	3	4	5	6	7	8	9	10			
10^{-5}	120	123	121	122	127	124	119	126	127	125	123.4	2.87	2.32
10^{-6}	11	11	9	11	12	9	12	9	9	12	10.5	1.35	12.89

Table 3d – Repeatability for Milk Analyst 4

Dilution	Repeatability										Mean	SD	% RSD
	1	2	3	4	5	6	7	8	9	10			
10^{-5}	119	128	124	124	119	120	126	121	126	119	122.6	3.40	2.78
10^{-6}	11	11	9	11	12	9	12	9	9	12	10.5	1.36	12.89

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Reproducibility.

The results for the analysis are demonstrated in the table below-

Dilution	Reproducibility																			
	Analyst 1		Mean	SD	%RSD	Analyst 2		Mean	SD	%RSD	Analyst 3		Mean	SD	%RSD	Analyst 4		Mean	SD	%RSD
10-5	122	124	122	2.82	2.31	124	119	121.5	3.53	2.94	120	119	119.5	0.70	0.59	123	117	120	4.24	3.53
10-6	12	10	11	1.41	12.85	12	9	10.5	2.12	20.20	12	13	12.5	0.70	5.65	9	10	9.5	0.70	7.44

Table 4a- Reproducibility for Chicken Breast

Table 4a- Reproducibility for Milk

Dilution	Reproducibility																			
	Analyst 1		Mean	SD	%RSD	Analyst 2		Mean	SD	%RSD	Analyst 3		Mean	SD	%RSD	Analyst 4		Mean	SD	%RSD
10-5	126	125	125.5	0.70	0.56	122	128	125	4.24	3.39	125	120	122.5	3.53	2.88	130	126	128	2.82	2.20
10-6	12	11	11.5	0.70	6.14	12	12	12	0	0	6	7	6.5	0.70	10.87	7	7	7	0	0

Accuracy

Accuracy of the given test method was analysed from the data obtained after the samples spiked with known concentration of 10^{-5} and 10^{-6} of *Escherichia coli* was tested.

The known concentration of the culture suspension is obtained by referring to the mean values of the culture suspension in **Table 1**

Accuracy is calculated as 88.69%

Limit of Detection

Limit of Detection for enumeration of *Enterobacteriaceae* was found to be 10 cfu/g

DISCUSSION

In the present study, the test parameter *Enterobacteriaceae* was validated by using two different food matrices by the test method ISO 21528 -2:2017. Two different matrix of food sample was tested by the same analysts under same laboratory conditions. Raw chicken and Milk sample were used for the entire study and the same spiked sample was used by the analysts to prevent more uncertainty in the testing. During the study the SD (standard deviation) and %RSD (Relative Standard Deviation) for the repeatability and reproducibility among the analysts was between the acceptable range. The analysis was performed by using an inoculum with low and high concentration of bacterial cells; and during the validation study it was observed that the test method ISO 21528-2:2017 was able to enumerate the *Enterobacteriaceae* when spiked in the food samples.

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CONCLUSION

In the present study Enumeration of *Enterobacteriaceae* in food sample by test method ISO 21528-2:2017 was validated. The study shows that low and high concentration of *Enterobacteriaceae* can be detected and enumerated, hence it is an efficient method which can be used for analysis of food samples.

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