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Implementation of ISO 16140-3:2021 for Enumeration of Enterobacteriaceae in Food Products

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ABSTRACT / ABSTRAK

Verifikasi metode merupakan salah satu langkah yang diperlukan untuk memastikan bahwa seluruh metode yang digunakan memenuhi standar yang diperlukan untuk keandalan dan keakuratan data pada laboratorium yang sudah menerapkan *Good Laboratory Practices* (GLP). Sesuai dengan ISO 16140-3:2021, verifikasi metode uji mikrobiologi dilaksanakan dengan menghitung Standar Deviasi Reproduksibilitas Intra Laboratorium (S_{IR}) untuk verifikasi penerapan metode (*implementation verification*) dan nilai eBias untuk verifikasi jenis pangan (*food item verification*). Verifikasi metode angka *Enterobacteriaceae* pada produk pangan menggunakan ISO 16140-3:2021 belum pernah dilakukan di laboratorium Badan POM. Sehingga verifikasi terhadap metode ini perlu dilakukan. Penelitian ini bertujuan untuk melakukan verifikasi terhadap ISO 21528-2:2017 sebagai acuan metode Angka Enterobacteriaceae pada produk pangan dengan teknik penghitungan koloni. Verifikasi penerapan metode menggunakan sampel susu pasteurisasi sedangkan untuk verifikasi jenis pangan, menggunakan sampel yogurt, telur pindang, permen manis dan keras, bumbu bubuk kering, kecap inggris dan keripik singkong. Seluruh sampel dicemari dengan bakteri Escherichia coli WDCM 00012. Nilai S_{IR} yang diperoleh adalah $0{,}12 \leq 2\times 0{,}18$ (rata-rata S_R terendah dari ISO 21528-2:2017), yang menunjukkan bahwa batas keberterimaan telah terpenuhi. Sedangkan nilai eBias kurang dari 0,5 $\log_{10}$, sehingga verifikasi metode Angka *Enterobacteriaceae* pada produk pangan yang dilakukan di Pusat Pengembangan Pengujian Obat dan Makanan Nasional (PPPOMN) Badan POM telah memenuhi syarat keberterimaan sesuai standar ISO 16140-3:2021.

Method verification is one of the steps required to ensure that all methods used meet the standards required for data reliability and accuracy in a laboratory that implements Good Laboratory Practices (GLP). In accordance with ISO 16140-3:2021, the method verification for microbiology testing implemented by calculating the Intralaboratory Reproducibility Standard Deviation (S_{IR}) for implementation verification and eBias value for food item verification. Method verification for enumeration of Enterobacteriaceae in food products using ISO 16140-3 has never been carried out at the Indonesian Food and Drug Authority (FDA) laboratories. Therefore, verification of this method needs to be performed. This study aimed to verify the ISO 21528-2:2017 as reference method for enumeration of Enterobacteriaceae in food products by colony count technique. The selected food item for implementation verification was pasteurized milk while for food item verification, the selected food items were yogurt, traditional preserved egg (telur pindang), sweet and hard candy, seasoning dry powder, worcester sauce and cassava chips. All of selected foods were artificially contaminated with Escherichia coli WDCM 00012. The S_{IR} value obtained was $0.12 \leq 2\times 0{,}18$ (lowest mean value of S_R from ISO 21528-2:2017), indicating that the acceptability limit for the

implementation verification was met. While the eBias value for all selected food item were less than 0,5 log10, thus the verification study in the National Quality Contol Laboratory of Drug and Food (NQCLDF), Indonesian FDA, meets the acceptance criteria defined in the standard ISO 16140-3:2021

Keywords: *eBias, food item, ISO 16140-3: 2021, microbiology, S_{IR}, verification*
Kata Kunci: eBias, jenis pangan, ISO 16140-3: 2021, mikrobiologi, S_{IR}, verifikasi

1. Introduction

Food is an indispensable part of everyday life. Consequently, food safety and quality are important factors supporting human health. Strict surveillance of pathogenic microorganisms in food products is required to prevent food-borne diseases from the consumption of contaminated food (Hoorfar, 2011). *Enterobacteriaceae*, a family of Gram-negative bacteria that includes various species such as *Salmonella*, *Escherichia coli*, and *Klebsiella*, are often identified as indicators of microbiological contamination of food products (Halkman & Halkman, 2014). The number of *Enterobacteriaceae* is directly correlated to the existence of pathogens in food products because this group of bacteria are utilized as failure indicators in the production process or of postproduction contamination (Silbernagel & Lindberg, 2002). Therefore, enumeration testing for *Enterobacteriaceae* in food products is important for the hygiene inspection (Paulsen et al., 2008). Food testing laboratories are required to be able to conduct enumeration methods of *Enterobacteriaceae* and perform verification to prove the ability of the laboratory to implement this method. Due to the quantitative interpretation involves colony-forming units (CFU) concepts, traditional culture technique is still applied as an international standard for microbiology (Feinberg et al., 2009).

In a laboratory that implements Good Laboratory Practices (GLP), method verification is one of the steps required to ensure that all methods used meet the standards required for data reliability and accuracy (Khezri et al., 2022). Testing laboratories that implement GLP can avoid discrepancies so that the resulting data is guaranteed to be valid and can be accounted for both legally and scientifically. Based on this statement, GLP is a management tool for laboratories to prevent nonconformities and improve and maintain the quality of test result data. As a management tool, GLP is not part of scientific knowledge but is an internal complement to laboratory practice to achieve qualified test result data (Faridah et al., 2018; International Organization for Standardization (ISO), 2017b).

The enumeration method of *Enterobacteriaceae* according to ISO 25128-2: 2017 is applicable for human consumption products, the feeding of animals, and environmental samples in the area of food production and food handling. This method stipulates VRBGA medium and biochemical identification of typical colonies (Baylis et al.,2011). Laboratory that have been accredited for ISO/IEC 17025: 2017, must validate or verify the method before using it for routine testing. One way to validate or verify the accuracy and reliability of *Enterobacteriaceae* testing is through the implementation of ISO 16140-3:2021, an international standard providing guidance and requirements for the validation and verification of microbiological testing methods in food (Belouafa et al., 2017; Leclercq, 2002; Lombard B, 2010; Lombard & Leclercq A, 2010). The standardization process was

established with approval from all parties and countries involved. These reference methods are generally based on conventional methods, allowing the main characteristics of a standard must be met (Lombard & Leclercq, 2011; Lombard & Leclercq A, 2010; Sandle, 2015).

The Intralaboratory Reproducibility Standard Deviation (S_{IR}) for implementation verification and eBias value for food item verification are parameters determined in the context of quantitative microbiological testing (International Organization for Standardization (ISO), 2019), particularly when adapting the ISO 16140-3:2021 standard for validation of alternative methods in a single laboratory. These parameters are essential for assessing the performance and reliability of an alternative method compared to a reference method. For each ISO method that includes the results of the validation study, implementation verification must be carried out prior to food item verification to prove that the user laboratory is able to implement the method successfully. If there is no validation study, laboratory users can only perform food item verification (ISO 16140-3:2021).

Verification of this method using ISO 16140-3 has never been carried out at the Indonesian FDA laboratories. Therefore, verification of this method needs to be performed. This study will determine the requirements for verifying the enumeration method of *Enterobacteriaceae* in food products according to the ISO 16140-3:2021 in the NQCLDF, Indonesian FDA. In general, the aims are to understand the verification procedures, the performance parameters that must be met, and the challenges and benefits associated with the implementation of this ISO method.

2. Metodology

2.1 Research Material

The food item for S_{IR} determination was selected from the scope of validation stated in ISO 21528-2:2017 Annex B (pasteurized milk). While the food item for eBias determination was selected based on various characteristics of matrix type stated in Indonesian FDA regulation No. 13/2019 (Table 1).

Culture media and confirmation reagent were prepared according to ISO 21528-2:2017 and the reference strain used was *Escherichia coli* WDCM 00012.

Table 1. The food items for verification studies for eBias determination

Food Item	Food Type	Food Category
Yogurt	Fermented milk drinks fermentation with flavored	Dairy products
Traditional preserved egg (*telur pindang*)	Egg product with heat-processed	Eggs and egg Products
Sweet and hard candy	Candy	Confectionary/Candy and Chocolate
Seasoning dry powder	Seasoning and condiment	Salt, spices, soups, sauces, salads, and protein products
Worcester sauce	Non-emulsion sauce	Salt, spices, soups, sauces, salads, and protein products
Cassava chips	Snacks based on potatoes, tubers, cereals, flour and starch	Ready-to-eat snack

(Indonesian FDA, 2019)

2.2 Preparation of inoculum and initial suspension

The reference strain was cultivated on an overnight TSA plate, and the suspension of 1 Mc Farland was made in NaCl 0,8% and diluted to obtain a combination of low, medium, and high-level concentrations. A sterile filter bag was filled with 25 grams of the sample. The sample was then mixed with 225 mL of PSS. A stomacher was used to homogenize the sample at 230 rpm for 30 seconds to achieve 1:10 dilution as described in ISO 6887-1:2017 and ISO 6887-4:2017.

2.3 Verification Method and Data Analysis

2.3.1 Implementation verification for S_{IR} determination

Ten prepared test portions in total were made duplo and contaminated in the initial suspension with varying concentrations of bacterial inoculum at low, medium, and high levels. The enumeration of *Enterobacteriaceae* was analyzed in accordance with ISO 21528-2:2017. The implementation verification was done simultaneously with two different technicians, media batches, and equipments (incubators, vortex mixers, pipettes).

The number of *Enterobacteriaceae* were calculated using the formula in accordance with ISO 16140-3:2021, to analyze the S_{IR} value. The calculated S_{IR} was compared to the result of the validation study (S_R values) taken from ISO 21528-2 Annex B (Table 2).

$$S_{IR} = \sqrt{\frac{1}{2n}\sum_{i=1}^{n}(y_{iA} - y_{iB})^2}$$

Table 2. Summary of S_R values from the validation study for ISO 21528-2 (ISO 16140-3:2021)

(Food) item	S_R values from the validation study			
	Low Inoculation Level	Medium Inoculation Level	High Inoculation Level	Mean value of three inoculation levels
Egg product	0.32	0.50	0.48	0.43
Raw meat	0.28	0.36	0.57	0.40
Animal feed	0.18	0.17	0.20	**0.18**
Pasteurized milk	0.24	0.18	0.19	0.20
Tiramisu	0.22	0.28	0.13	0.21

2.3.2 Food item verification for eBias determination

The food items were artificially contaminated at three different levels of inoculation in the initial suspension. Each level was performed in duplicate. The artificially contaminated food items and the inoculum suspension used to inoculate the food items were enumerated using ISO 21528-2:2017. The analysis was carried out under replicability conditions by one technician at the same time.

In compliance with ISO 16140-3:2021, an eBias analysis was carried out. The absolute difference between the log numbers of *Enterobacteriaceae* from contaminated food items and bacterial inoculum suspension was used to analyze the estimated Bias (eBias) value.

3. Result and Discussion

The number of *Enterobacteriaceae* (calculated as cfu/g in test portions A and B of laboratory sample) in contaminated pasteurized milk sample is shown in Table 3. The calculated S_{IR} was 0.12 while the S_R value from the validation study taken from ISO 21528-2 Annex B was 0.18.

Tabel 3 Calculation of S_{IR}

Laboratory sample number	Result A	Result B	Log_{10} result A yiA = log_{10}(xiA)	Log_{10} result B yiB = log_{10}(xiB)	Absolute difference |yiA – yiB|	Squared difference |yiA – yiB|^2
1	155	140	2.19	2.15	0.0442	0.0020
2	100	120	2.00	2.08	-0.0792	0.0063
3	120	170	2.08	2.23	-0.1513	0.0229
4	95	135	1.98	2.13	-0.1526	0.0233
5	435	710	2.64	2.85	-0.2128	0.0453
6	325	595	2.51	2.77	-0.2626	0.0690
7	380	505	2.58	2.70	-0.1235	0.0153
8	3200	4950	3.51	3.69	-0.1895	0.0359
9	3500	6050	3.54	3.78	-0.2376	0.0565
10	3000	6050	3.48	3.78	-0.3046	0.0928
					SUM	0.2763
					Sum/(2xn)	0.0153
					S_{IR}	**0,12**

Tabel 4. Result of S_{IR} compare to S_R value from ISO 21528-2:2017

Food item	S_{IR} Value	Mean value of S_R ISO 21528-2:2017
Pasteurized milk	0.12	0.18

Implementation verification for quantitative methods is achieved by determining the intralaboratory reproducibility standard deviation, expressed as S_{IR}. ISO 16140-3 noted that the S_{IR} of the verified method shall be less than or equal to twice the lowest mean value of the reproducibility standard deviation (S_R) of the (food) items used in the validation study stated in ISO 21528-2: 2017. When only one S_R value was assigned in the validation study, the S_{IR} of the verified method should be less than or equal to twice the lowest mean value of the S_R value.

Implementation verification must be performed prior to food item verification. Implementation verification only requires the S_{IR} value. The S_{IR} was designed on a minimum of 10 laboratory samples. Implementation verification was conducted in a single laboratory, and the reproducibility was yielded from two different media batches, technicians, and equipment (incubators, pipettes, vortex mixers) (International Organization for Standardization (ISO), 2021). Factors that might result variation in microbiological methods for food analysis include laboratory method, error in dilution or inhomogeneity of the test materials (Ellison., et.a; 2012).

One (food) item within the scope of validation was used for the implementation

verification. According to ISO 21528-1, validation studies were carried out on egg products, raw meat, animal feed, pasteurized milk, and tiramisu (Table 2). The implementation of S_{IR} parameters in this study used pasteurized milk. As shown in Table 3, the S_{IR} obtained in this study was 0.12, while the lowest mean value obtained from ISO 21528-2:2017 was 0.18. The value of 0.18 is less than twice of 0.12 value, hence, the results meet the acceptability requirements. This result indicated that the NQCLDF could implement ISO 21528-2:2017 appropriately.

The food item selected for implementation verification greatly influences the deviation value. The easier to homogenize, the smaller the deviation. If the sample used is very heterogeneous, sample preparation and technical competency are expected to reduce deviations in implementation verification. In this study, pasteurized milk tends to be homogenous and included within the scope of validation and surveillance of the Indonesian FDA. The contamination levels used shall be representative of the range of the natural contamination found in the laboratory samples. The range of inoculum was in the range of 80 CFU/mL to 4000 CFU/mL to cover the requirements for pasteurized milk stated in the Indonesian FDA Regulation No. 13/2019 about The Maximum Limit of Microbial Contamination in Processed Food.

Table 5. The eBias Results from Food Item Verification

Food Items	eBias Values		
	Low	Medium	High
Yogurt	0.02	0.11	0.08
Traditional preserved egg (telur pindang)	0.03	0.00	0.00
Sweet and hard candy	0.07	0.02	0.01
Seasoning dry powder	0.13	0.12	0.08
Worcester sauce	0.08	0.02	0.13
Cassava chips	0.05	0.09	0.05

Apart from implementation verification, food item verification must also be performed. The eBias parameter is only required for (food) item verification. In accordance with ISO 16140-3: 2021, the number of food items used in the verification depends on the scope of the method to be developed. The larger the scope, the more food items are required to be validated.

Based on ISO 16140-3: 2021, to make a larger scope of method in food products as general, it requires at least 5 food items to be verified. For food verification purpose, this study used 6 items of food from different food categories that have Enumeration of *Enterobacteriaceae* as the test parameters in accordance with the Indonesian FDA Regulation No.13/2019, as shown in Table 5. Those 6-food items were chosen due to their challenging characteristics as suggested in ISO 16140-3: 2021.

The scope of food surveillance carried out by the Indonesian FDA covers all categories of processed food. Therefore, finding naturally contaminated samples was so difficult that artificial contamination was the only option. The reference cultures used were commercially obtained with unknown values, so the inoculum preparation is one of the critical points in conducting verification. The inoculum preparation during the preliminary test and verification must have similar inoculum levels to prevent differences in the amount of

inoculum during verification.

It was expected that, at each level, the absolute difference between the results from the artificially contaminated food and that of the inoculum suspension is less than or equal to 0.5 $\log_{10}$. The results in Table 5 indicated that the eBias value between 6 food items was varied, nonetheless at each level of contamination the absolute difference between the two results is less than 0.5 $\log_{10}$, then the quantitative method for *Enterobacteriaceae* to be verified works correctly in the user laboratory.

In future verification for quantitative methods, the measurement uncertainty of the S_{IR} value should be determined simultaneously as it is more visible to minimize the factors that affect the raising of uncertainty values. The smaller measurement uncertainty, the more reliable the laboratory performance is, thus consumers trustworthy will be augmented.

4. Conclusion

According to the results obtained, the NQCLDF - Indonesian FDA successfully implemented ISO 21528-2: 2017 and proved that the laboratory could perform quantitative methods for *Enterobacteriaceae* on an extensive range of food products. The results have comprehensively elucidated the method's performance characteristics according to laboratory needs. This study has followed the protocol in ISO 16140-3:2021 to determine the performance of implementation verification and food item verification. The acceptance criteria of each parameter have also been clearly stated in ISO 16140-3:2021. Hence, this study is fruitful for the microbiology laboratory to have a better understanding on implementing ISO 16140-3:2021. However, the protocols sometimes emerge ambiguity that leads to raising so many perceptions that there are differences in the way of testing and analyzing the results. Despite the satisfactory results, practically, personnel competency plays an important role, and preparing the inoculum, carrying out homogenization, and sample preparation are still the biggest challenging steps in this study.

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