Performance Characteristics of the Quantitative Method for *Staphylococcus aureus* in Food Products corresponds to ISO 16140-3: 2021

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ABSTRACT / ABSTRAK

Good food sanitation is one of the main pillars for achieving food security goals. High levels of *Staphylococcus aureus* in food can indicate poor hygiene and handling practices during food production, processing, or storage. Before testing, the laboratory needs to verify the quantitative method for *Staphylococcus aureus* to produce valid data to ensure food safety and quality. The Centre of National Quality Laboratory of Drugs and Food, Indonesian Food and Drug Authority (FDA) has never verified the *Staphylococcus aureus* quantification method based on the latest ISO 16140-3:2021. Following the guidelines established in ISO 16140-3:2021, method verification is accomplished by calculating the Interlaboratory Reproducibility Standard Deviation (S_{IR}) for implementation validation and the eBias value for verifying the method's suitability for assessing specific food items. This research was conducted to confirm the ISO 6888-1:2021 as the designated reference method for quantifying *Staphylococcus aureus* in food products. The cheese was utilized as the test food item to verify the implementation of the method. At the same time, several products, including condensed milk, margarine, baby porridge, cassava chips, and ready-to-eat sausage, were examined as challenging food types. Every chosen food item was artificially contaminated with *Staphylococcus aureus* WDCM 00034. The S_{IR} value obtained was $0.04 \leq 2\times 0.11$ (the lowest mean of SR value from ISO 6888-1:2021), which indicated that the Centre of National Quality Laboratory of Drugs and Food Indonesian FDA was able to implement the method very well. In addition, the eBias value for all types of food tested was below 0.5log10, which showed that the quantitative method for coagulase-positive *Staphylococci* (*Staphylococcus aureus* and other types) could be applied in the Centre of National Quality Laboratory of Drugs and Food laboratory for the extensive scope of food.

Sanitasi pangan yang baik merupakan salah satu pilar utama untuk mencapai tujuan food security. Tingginya jumlah Staphylococcus aureus dalam makanan dapat mengindikasikan buruknya sanitasi selama proses produksi atau penyimpanan pangan. Sebelum melakukan pengujian, sangat penting bagi laboratorium untuk melakukan verifikasi terhadap metode kuantitatif Staphylococcus aureus guna menghasilkan data yang valid dalam rangka memastikan keamanan dan kualitas pangan. Laboratorium Pusat Pengembangan Pengujian Obat dan Makanan Nasional (PPPOMN) Badan Pengawas Obat dan Makanan (BPOM) belum pernah melakukan verifikasi metode kuantifikasi Staphylococcus aureus berdasarkan ISO terbaru 16140-3:2021. Sesuai dengan pedoman yang ditetapkan dalam ISO 16140-3:2021, verifikasi metode dilakukan melalui penghitungan, yakni Standar Deviasi Reprodusibilitas Intralaboratorium

(SIR) untuk verifikasi implementasi dan nilai eBias untuk memverifikasi kesesuaian metode dalam menilai berbagai jenis pangan. Penelitian ini dilakukan dengan tujuan mengkonfirmasi ISO 6888-1:2021 sebagai metode acuan yang ditetapkan dalam menghitung jumlah Staphylococcus aureus pada pangan olahan. Keju digunakan sebagai sampel uji untuk verifikasi implementasi di laboratorium, sedangkan sejumlah produk termasuk susu kental manis, margarin, bubur bayi, keripik singkong, dan sosis siap makan digunakan sebagai jenis pangan yang menantang dalam rangka memverifikasi jenis pangan. Setiap jenis produk pangan yang digunakan dalam verifikasi, dicemari Staphylococcus aureus WDCM 00034. Nilai SIR yang diperoleh adalah 0.04 ≤ 2× 0.11 (rata-rata nilai SR terendah dari ISO 6888-1:2021) yang menandakan laboratorium PPPOMN Badan POM mampu mengimplementasikan metode dengan baik. Selain itu, nilai eBias untuk semua jenis pangan yang diujikan berada di bawah 0.5log10, yang menunjukkan bahwa metode kuantitatif untuk Staphylococci positif koagulase (Staphylococcus aureus dan jenis lainnya) dapat digunakan di Laboratorium PPPOMN dalam ruang lingkup pangan yang lebih luas.

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

1. Introductions

Staphylococcus aureus is a potential foodborne pathogen. It can produce toxins that cause food poisoning when ingested. Therefore, the presence of this bacterium in food products can pose a significant food safety risk (Fetsch & Johler, 2018). Consuming food contaminated with *Staphylococcus aureus* can lead to foodborne illnesses, including symptoms such as nausea, vomiting, diarrhea, and abdominal cramps (CDC, 2023). The severity of these symptoms can vary depending on the level of contamination and the individual's susceptibility, potentially leading to hospitalization in severe cases (Hennekinne et al., 2012). The impact of these illnesses can be severe, depending on the amount of toxin produced and the individual's immune response, making it crucial to prevent *Staphylococcus aureus* contamination in the food supply chain (Kadariya et al., 2014). High levels of *Staphylococcus aureus* in food can indicate poor hygiene and handling practices during food production, processing, or storage (Gutiérrez et al., 2012). Outbreaks of *Staphylococcus aureus* food poisoning have been reported worldwide, underlining the need for rigorous monitoring and control measures (Balaban & Rasooly, 2000).

Many regulatory agencies and governments have established maximum allowable limits for *Staphylococcus aureus* in certain food products. In Indonesia, the maximum permissible limits for *Staphylococcus aureus* in food products are regulated in Indonesian FDA Regulation No. 13, 2019. Enumeration testing helps determine the level of *Staphylococcus aureus* contamination and assess the potential consumer risk. Monitoring the enumeration of this bacterium is an essential quality control measure to ensure that food products meet specific quality and safety standards (International Organization for Standardization (ISO), 2021a). Strict surveillance of pathogenic microorganisms in food products is necessary to prevent food-borne diseases from consuming contaminated food (Hoorfar, 2011). It is essential to ensure compliance with these legal requirements. To maintain food safety and quality, verifying the quantitative method of *Staphylococcus aureus* is very important. According to Wang et al. (2022), global food safety requires continuous monitoring of pathogenic microorganisms to prevent foodborne illnesses and ensure consumer health.

Method verification is a critical step in ensuring that all methods used, especially in laboratory testing and research, meet the standards required for data reliability and accuracy (Abdel & El-Masry, 2021). It ensures that a particular testing or analytical method can produce accurate and precise results. This means the method's measurements are close to the actual value, and repeated measurements under the same conditions yield consistent results (Sushila Dagadu Chavan & Deepa Mahendra Desai, 2022). Method verification is a critical component of quality control in laboratories. It helps monitor and maintain the quality and reliability of testing processes, ensuring that the methods are suitable for their intended purpose (Taverniers et al., 2004). In situations where different laboratories may use the same method, method verification helps ensure that the results are comparable and that the method is applied consistently across various settings. Moreover, method verification across multiple laboratories helps ensure consistency in results, which is particularly important in the global food trade where standardized practices must be followed (Elliott et al., 2020).

ISO 6888-1:2021 provides guidelines for enumerating coagulase-positive *Staphylococci* (*Staphylococcus aureus* and other species) using microbiological culture methods. The principle is to take a food or feed product sample, inoculate it into a suitable growth medium, and count the bacterial colonies that grow after a certain incubation period. Referring to the guidelines set out in ISO 16140-3:2021 is a critical approach to ensure the accuracy and reliability of the quantitative method for *Staphylococcus aureus* in food products. ISO 16140-3:2021 is a specific part of the ISO 16140 series that guides validating alternative (proprietary) methods for microbiological testing. The standard provides a structured process for verifying proprietary testing methods against a reference method. This verification ensures that alternative methods are as reliable as established reference methods (International Organization for Standardization (ISO), 2021b). According to Zhang et al. (2020), the standardization of microbiological methods like ISO 16140-3:2021 is essential to ensuring the safety of food products globally.

There are two parameters used in the context of quantitative microbial testing: the Interlaboratory Reproducibility Standard Deviation (S_{IR}) for implementation verification and the eBias value for food item verification (International Organization for Standardization (ISO), 2019). By evaluating these performance characteristics, laboratories can verify and validate their quantitative methods for *Staphylococcus aureus* in food products, ensuring that the methods are accurate, reliable, and suitable for their intended purposes, as recommended by ISO 16140-3:2019.

This study will present the general procedure of verifying the enumeration method of coagulase-positive *Staphylococci* and assign the performance characteristics referred to ISO 16140-3: 2021. Moreover, this study will also outline the critical criteria supporting the method verification's fruitfulness.

2. Methodology

2.1 Research Material

The reference strain *Staphylococcus aureus* WDCM 00034 was purchased from Microbiologics, USA. Cultural media purchased from Merck, Germany, prepared by ISO 6888-1:1999+A2:2018. The sample used for implementation verification (S_{IR} determination) is the type of food selected from the scope of validation in ISO 6888-1:1999+A2:2018 Annex A (Cheese). Food item verification (eBias determination) uses

samples from several food items selected based on various characteristics of matrix types stated in Indonesian FDA regulation No. 13/2019 (Table 1).

Table 1. Food Items Intended for Verification Studies Aimed at Determining eBias (Indonesian FDA, 2019)

Food Item	Food Type	Food Category
Condensed Milk	Condensed Milk and Its Analogues	Dairy products and their analogs
Margarine	Fat Emulsions, Especially Water-in-Oil Emulsion Type	Fats, Oils, and Oil Emulsions
Baby Porridge	Food for babies and growing children	Processed food for particular nutritional needs
Cassava Chips	Snacks made from potatoes, tubers, cereals, flour, or starch (from tubers and nuts)	Ready-to-eat snacks
Ready-to-eat Sausage	Processed meat products, poultry meat, and ground game meat	Meat and meat products, including poultry and game

2.2 Preparation of Reference Strain as Artificial Contamination and Initial Suspension

The results of cultivating the reference strain on an overnight Tryptic Soy Agar (TSA) plate were prepared to make a 0.8% NaCl suspension with a turbidity of 1 Mc Farland. Serial dilution made The suspension into low, medium, and high-level concentrations. A sterile filter bag was filled with 25 grams of the sample. The sample was mixed with 225 mL of Peptone Salt Solution (PSS). A stomacher homogenized the sample at 230 rpm for 30 seconds to achieve 1:10 dilution.

2.3 Verification Method

2.3.1 Implementation Verification for S_{IR} Determination

Ten test portions were prepared in duplicate and deliberately contaminated in the initial suspension with different levels of bacterial inoculum, including low, medium, and high concentrations. The quantification of *Staphylococcus aureus* followed the guidelines outlined in ISO 6888-1:2021. Two technicians conducted The verification process simultaneously using two different batches of media and equipment, including incubators, vortex mixers, and pipettes.

2.3.2 Food Item Verification for eBias Determination

The food products underwent artificial contamination with inoculums at three distinct levels in the initial suspension, with each level tested in duplicate. Enumeration of the artificially contaminated food items and the inoculum suspension used for contamination was performed per ISO 6888-1:2021. The analysis was conducted simultaneously under replicable conditions by a single technician.

Following the guidelines outlined in ISO 16140-3:2021, an eBias evaluation was conducted. The eBias value was assessed by calculating the absolute difference between the logarithmic counts of *Staphylococcus aureus* in contaminated food items and the bacterial inoculum suspension.

2.4 Data Analysis

2.4.1 Implementation Verification for S_{IR} Determination

The number of *Staphylococcus aureus* was calculated using the formula by ISO 16140-3:2021 to analyze the intralaboratory reproducibility standard deviation (S_{IR}) values.

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

2.4.2 Food Item Verification for eBias Determination

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

3. Results and Discussion

Verification of the enumeration of coagulase-positive *Staphylococci* (*Staphylococcus aureus* and other species) has been carried out by ISO 16140-3: 2021 by determining the Interlaboratory Reproducibility Standard Deviation (S_{IR}) parameters for implementation verification and eBias for food item verification. The validation study on ISO 6888-1:1999+A2:2018 used three types of food: cheese, meat, and egg powder, with the lowest average S_R value of egg powder (0.11) (Table 2). Implementation verification in this study was carried out using cheese samples with a S_{IR} value of 0.04 (Table 3 and Table 4).

For implementation verification, the S_{IR} of the verified method shall be equal to or less than twice the lowest mean value of the reproducibility standard deviation (S_R) of the (food) items used in the validation study stated in ISO 6888-1:2021. This study noted that *the* S_{IR} value for cheese was less than twice the average S_R value for egg powder, which, this indicates that the laboratory was able to implement the method very effectively.

Table 2. Summary of S_R values from the validation study for ISO 6888-1

(Food) item	S_R values from the validation study			
	Low Inoculation Level	Medium Inoculation Level	High Inoculation Level	The mean value of three Inoculation levels
Cheese	0.19	0.16	0.24	0.20
Meat	0.17	0.17	0.14	0.16
Egg Powder	0.11	0.10	0.11	**0.11**

Table 3. Result of S_{IR} Compared to S_R Value from ISO 6888-1:2021

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	550	570	2.74	2.76	-0.0155	0.0002
2	520	590	2.72	2.77	-0.0548	0.0030
3	5900	5700	3.77	3.76	0.0150	0.0002
4	6,200	5,500	3.79	3.74	0.0520	0.0027
5	6,300	6,100	3.80	3.79	0.0140	0.0002
6	11,000	11,000	4.04	4.04	0.0000	0.0000
7	10,000	10,000	4.00	4.00	0.0000	0.0000
8	13,000	9,900	4.11	4.00	0.1183	0.0140
9	43,000	55,000	4.63	4.74	-0.1069	0.0114
10	46,000	56,000	4.66	4.75	-0.0854	0.0073
					SUM	0.0391
					Sum/(2xn)	0.0020
					S_{IR}	**0.0442**

Table 4. The result of S_{IR} compared to the S_R value from ISO 6888-1:2021

Food item	S_{IR} Value	The mean value of SR ISO 6888-1:2021
Cheese	0.04	0.11
Acceptance Criteria		$S_{IR} \leq 2 \times 0.11$
		0.04 < 0.22 (comply with the requirement)

The verified method is for counting *Staphylococcus aureus* and other *Staphylococci* that can coagulase plasma. The coagulase test is one of the methods used to differentiate highly pathogenic *Staphylococcus aureus* from other less pathogenic *Staphylococcal* species (Chamberlain, 2009). This study used *Staphylococcus aureus* WDCM 00034 to contaminate the samples artificially due to the difficulties of finding cheese naturally contaminated with *Staphylococcus aureus*.

Table 5. The eBias Results from Food Item Verification

Food Item	eBias Value		
	Low	Medium	High
Condensed Milk	0.09	0.02	0.01
Margarine	0.00	0.05	0.07
Baby Porridge	0.05	0.19	0.01
Cassava Chips	0.05	0.17	0.12
Ready-to-eat Sausage	0.18	0.13	0.07

The successful implementation verification using cheese, as demonstrated by the low SIR value, underscores the robustness of the ISO 6888-1:2021 method in detecting *Staphylococcus aureus*. The importance of low SIR values cannot be overstated as they reflect the method's reproducibility, which is crucial for ensuring consistent results across different laboratories (Latimer, 2023). This consistency is particularly vital in the global food

industry, where standardized methods are necessary to maintain food safety and ensure compliance with international regulations (Elliott et al., 2020).

Food item verification compared artificially contaminated samples and inoculum control. In this study, five types of food were used to cover the extensive scope of food products. These five types of food have represented challenging food, for example, samples with a high-fat content (margarine), high sugar level (condensed milk), and high microbiota content (baby porridge, cassava chips, and ready-to-eat sausage).

Food matrices can vary significantly in composition, including fat content, sugar levels, microbiota, pH, and more (Aryani et al., 2016). By selecting food items representing a broad spectrum of these characteristics, the verification process becomes more representative of real-world scenarios. Different food matrices can pose unique challenges to testing methods. For example, high-fat content in some foods can interfere with microbial detection, and high-sugar foods can impact the growth of microorganisms (Hamad, 2012). By including a variety of matrices, the verification process can evaluate the method's ability to handle these challenges and provide accurate results in a range of conditions. By testing the method across different matrices, it can be confirmed that the method is specific to the target microorganism and not unduly influenced by the matrix components (Latimer, 2023). The ability of the method to produce consistent and accurate results, regardless of the food matrix, demonstrates its robustness and versatility, making it a valuable tool for food safety laboratories worldwide. This study's findings also emphasize the importance of using well-characterized reference materials and adhering to strict biosafety protocols, especially when working with pathogenic microorganisms like *Staphylococcus aureus* (Kadariya et al., 2014).

According to ISO 16140-3:2021, the absolute difference between the artificially contaminated food and the inoculum suspension as positive control is equal to or less than 0.5 log10. The results in Table 5 showed that the eBias value between five food items was diverse and complied with the requirement. From those results, it was noted that each level of contamination between the absolute difference between the contaminated samples and positive control was less than 0.5 log10. In conclusion, the user laboratory successfully verified the enumeration of coagulase-positive Staphylococci.

Considering several factors that may contribute to the verification process, this study used well-characterized and purified reference materials to prevent non-conformities. In this case, using commercial references should be considered. Moreover, proper biosafety practices should be applied since coagulase-positive *Staphylococci* are known as pathogenic bacteria. Another factor identified during this study was how to appropriately treat challenging food as the representatives for food item verification based on ISO 16140-3: 2021. Those challenging food samples should be prepared and homogenized according to ISO 6887 (all parts): 2017. In addition, fully understanding the essential statistical element applied in ISO 16140-3: 2021 is also essential in this study because the data analysis sometimes needs conversion, especially for the test portion.

The enumeration of *Staphylococcus aureus* in this verification process is done meticulously using the standardized formula outlined in ISO 16140-3:2021. ISO 16140-3:2021's stringent guidelines emphasize the analysis of interlaboratory reproducibility standard deviation (S_{IR}) and eBias values, which are crucial for assessing consistency across different laboratories. Careful S_{IR} calculations and analysis guarantee accuracy and

reproducibility, enhancing the method's reliability and integrity. This meticulous adherence to international standards instills confidence in the verification process's ability to accurately detect and count *Staphylococcus aureus*, a critical step in ensuring food safety and quality.

Nonconformities may occur during verification, and personnel must be competent to identify the problem. This proactive approach not only rectifies immediate discrepancies but also acts as a preventative measure to ensure that similar problems do not recur. Moreover, when verifying quantitative methods, it is essential to assess the measurement uncertainty of the S_{IR} value concurrently to reduce the factors that can increase uncertainty. This approach makes measurement uncertainty more evident and enhances the reliability of laboratory performance, thereby increasing consumer trust.

4. Conclusion

This work discussed how to meet the requirements of performance characteristics of the enumeration method for coagulase-positive *Staphylococci* (*Staphylococcus aureus* and other species) by ISO 16140-3: 2021. Both implementation verification and food item verification showed satisfactory results, meaning the user laboratory could implement the ISO 6888-1: 2021 well. In line with implementation verification, food item verification provided satisfactory results, reflecting an accurate and reliable method to be applied in the user laboratory. Since Centre of National Quality Laboratory of Drugs and Food Indonesian FDA has been equipped with calibrated instruments, competent personnel, and pure references, this study's SIR and eBias value complied with the verification criteria. In conclusion, this meticulous verification process, as outlined in ISO 16140-3, ensures that laboratories are well-prepared to maintain the highest standards of accuracy and reliability when enumerating coagulase-positive *Staphylococci*, ultimately contributing to enhanced food safety and quality control.

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