Optimization and Validation of Analytical Method for Detection of *Shigella* sp. in Oral Preparations of Quasi-Drugs in the Form of Lozenges

Desty Herawati [a,1,*], Eko Aprianto[b,2], Eni Cahyaningsih[a,3], Nur Miftahurrohmah[c,4]

[a]Indonesian Food and Drug Authority, Jl. Percetakan Negara No.23, Jakarta Pusat, 10560, Indonesia
[b]Indonesian Food and Drug Authority Regional Office in Banda Aceh, Indonesian Food and Drug Authority, Jl. Daud Beureueh No.110, Banda Aceh, 23126, Indonesia
[c]Microbiology Laboratory, Faculty of Pharmacy, Universitas Pancasila, Srengseng Sawah, Jagakarsa, Jakarta Selatan, 12640, Indonesia
[1]desty.herawati@pom.go.id*; [2]eko.aprianto@pom.go.id; [3]eni.cahyaningsih@pom.go.id; [4]nur_miftahurrohmah@univpancasila.ac.id
*corresponding author

ARTICLE INFO

Article history
Received:
October 31, 2024

Revised:
September 21, 2024

Accepted:
October 02, 2024

DOI:
https://doi.org/10.54384/eruditio.v4i2.199

ABSTRACT / ABSTRAK

Lozenges, a quasi-drug used to relieve sore throats, must meet the safety and quality requirements according to Indonesian FDA Regulation No. 7 of 2023 concerning the criteria and procedures for quasi-drug registration, which stipulates that oral preparations of quasi-drugs must be free from *Shigella* spp—microbial contamination. Contamination by *Shigella* bacteria can cause diseases such as shigellosis, characterized by symptoms such as diarrhoea, vomiting, and fever. This study aims to validate the *Shigella* spp—detection method in quasi-drug lozenges using the WHO (Quality Control Methods for Herbal Materials) reference method. Validation was carried out to ensure that this method can be used for routine analysis in the Indonesian FDA laboratory. The guidelines used to validate the microbiological method are the Singapore Accreditation Council (SAC) Guidance Notes C&B AND ENV 002 of 2019 and refer to pharmacopoeias. The study results showed that the WHO (Quality Control Methods for Herbal Materials) method in detecting *Shigella* sp. has a sensitivity and specificity of 100%, with false positive and false negative rates of 0% each. The detection limit obtained was 3 cfu/g, which meets the requirements (below 10 cfu/g). The method suitability test (optimization) showed that the initial solvent used for sample homogenization in *Shigella* detection was sufficient using Tryptone Soya Broth (1:10) without additional neutralization or modification procedures at the sample homogenization stage. Based on these results, the *Shigella* spp. The WHO reference (Quality Control Methods for Herbal Materials) detection method is accurate. It can be applied for routine microbial contamination testing analysis in the Food and Drug Supervisory Agency laboratories.

Tablet hisap obat kuasi yang digunakan untuk meredakan sakit tenggorokan harus memenuhi persyaratan keamanan dan mutu sesuai Peraturan Badan POM No. 7 Tahun 2023 tentang kriteria dan tata laksana registrasi obat kuasi, yang menetapkan bahwa sediaan oral obat kuasi harus bebas dari cemaran mikroba Shigella spp. Kontaminasi oleh bakteri Shigella dapat menyebabkan penyakit seperti shigellosis yang ditandai dengan gejala seperti diare, muntah, dan demam. Penelitian ini bertujuan untuk memvalidasi metode deteksi Shigella spp. pada tablet hisap obat kuasi menggunakan metode acuan WHO (Quality Control Methods for Herbal Materials). Validasi dilakukan untuk memastikan metode ini dapat digunakan untuk analisis rutin di laboratorium BPOM. Pedoman yang digunakan untuk memvalidasi metode mikrobiologi adalah Singapore Accreditation Council (SAC) Guidance Notes C&B AND ENV 002 Tahun 2019 serta farmakope. Hasil penelitian menunjukkan bahwa metode WHO (Quality

Control Methods for Herbal Materials) dalam mendeteksi Shigella sp. memiliki sensitivitas dan spesifisitas sebesar 100%, dengan tingkat positif palsu dan negatif palsu masing-masing 0%. Limit deteksi yang diperoleh adalah 3 cfu/g, yang memenuhi persyaratan (di bawah 10 cfu/g). Dari uji kesesuaian metode (optimasi) yang dilakukan menunjukkan bahwa pelarut awal yang digunakan untuk homogenisasi sampel pada deteksi Shigella cukup menggunakan Tryptone Soya Broth (1:10) tanpa perlu prosedur penetralan atau modifikasi tambahan pada tahapan homogenisasi sampel. Berdasarkan hasil ini, metode deteksi Shigella spp. menggunakan metode acuan WHO (Quality Control Methods for Herbal Materials) dinyatakan akurat dan dapat diterapkan untuk analisis rutin pengujian cemaran mikroba di laboratorium-laboratorium Badan Pengawas Obat dan Makanan.

Keywords: *Method validation, Shigella, quasi-drug, lozenges, microbiological examination of non-sterile product, SAC-Singlas*
Kata Kunci: Validasi metode, *Shigella,* obat kuasi, tablet hisap, pengujian mikrobiologi produk nonsteril, SAC-Singlas

1. Introduction

According to Indonesian FDA regulation No.7 of 2023, quasi-drugs are preparations containing active ingredients with pharmacological effects that are non-systemic or local to treat minor complaints. Quasi-drug preparations consist of 2 types, namely topical and oral. Various kinds of quasi-medicinal products are circulating in Indonesia, so monitoring these products' physical, chemical and biological safety aspects is necessary. Quasi-drugs circulating in Indonesia must meet the safety and quality requirements of finished quasi-drug products listed in the latest Indonesian FDA regulation No.7 of 2023 concerning criteria and procedures for registration of quasi-drugs. One of the requirements in the regulation states that oral quasi-medicinal products must not contain *Shigella* contamination.

Based on the website (https://cekbpom.pom.go.id/obat_kuasi, accessed on October 23, 2023), the Indonesian FDA has issued 12 quasi-medicinal products in the form of lozenges. Adults and children widely use quasi-medicinal lozenges to treat minor throat complaints (Rathod et al., 2018). Quasi-medicinal lozenges commonly circulated in the market are generally complex candy types, also known as lozenges. Hard candy-type lozenges have hygroscopic properties, so if not packaged properly, they will increase the water content in the lozenges, which can potentially cause the growth of microorganisms (Choursiya & Andheriya, 2018).

Microbial contamination in quasi-medicinal products can potentially reduce or even activate therapeutic activity and harm one's health (Aini et al., 2021). *Shigella* bacteria are capable of producing toxins and causing diseases such as dysentery, diarrhoea, and even death. *Shigella* is a gram-negative bacterium in the *Enterobacteriaceae* family that causes Shigellosis (Muzembo et al., 2023). Shigellosis is a disease caused by *Shigella* species bacteria that infect the intestinal tract and rectum (Kotloff et al., 2018). In a study in East Africa, *Salmonella* and *Shigella* were the main pathogenic bacteria most commonly reported as contaminants in traditional medicines (Walusansa et al., 2021). *Shigella* was a bacterial contaminant in traditional medicine samples studied in Nairobi, Kenya (Korir et al., 2017). *Shigella*e are highly infectious; the infective dose is about 103 organisms compared to 105-108 for *Salmonella* and Vibrio (Brooks et al., 2012).

Quasi-medicinal products before circulating to the market and those that have circulated in the market must meet safety, efficacy and quality requirements. One safety requirement

that oral quasi-drugs must meet is that they must not contain *Shigella* microbial contamination; this can be proven through *Shigella* detection testing in the laboratory. Therefore, the laboratory needs a validated analytical method to detect microbial contamination. Method validation is an essential factor in obtaining analytical results that are valid, reliable, and can be scientifically accounted for by the intended use (Faridah et al., 2018).

In 2023, the Food and Drug Administration did not have a test method for detecting *Shigella* quasi-drugs in lozenges. Indonesian FDA only has a validated *Shigella* detection analysis method (MA PPOMN No.52/MI/15) for testing traditional medicine samples because there were no regulations requiring microbial contamination limits for finished quasi-medicine products. According to ISO 17025:2017, the laboratory must validate the reference method used if it is used outside the intended scope, outside its designation, or modified. In this study, the WHO issued the reference method for *Shigella* detection analysis on quality control methods for herbal materials (World Health Organization, 2011). The method issued by WHO is intended for herbal/traditional medicine samples, so if the laboratory wants to apply the technique to quasi-medicine samples, validation testing of the method is needed before use.

This study focuses on validating *Shigella* sp detection methods in quasi-medicinal products in the form of lozenges using conventional culture-based methods based on the methods recommended by WHO. The selection of this method considers various factors such as cost, equipment, reagents/media, human resources, as well as accommodation and environmental conditions in the Indonesian FDA Provincial Offices throughout Indonesia. In addition, based on the United States Pharmacopeia Convention (USP) 2022 in appendix <1223>, if there are differences in test results between alternative and conventional microbiological methods, the traditional/culture method is still used as a gold standard reference.

However, recent developments have shown that several alternative molecular based (DNA) methods offer higher speed and accuracy in detecting *Shigella*. For example, a study by Yang et al. (2020) compared the performance of conventional PCR, real-time PCR, and droplet digital PCR (ddPCR) in detecting *Shigella* in food, where ddPCR showed the highest sensitivity with a detection limit as small as 0.1 cfu/mL. Another rapid method is real-time Loop-Mediated Isothermal Amplification (LAMP), which, according to a study by Liew et al. (2014), can detect *Shigella* within 90 minutes. Still, it has a relatively large detection limit of 5.9 x 10^5 cfu/mL.

In this study, the culture-based WHO method will be validated to prove that the process meets the requirements of the predetermined validation parameters. This study aims to produce a valid analytical method to detect *Shigella* contamination in quasi-oral drug samples in the form of lozenges. A valid analytical process is expected to protect the public from products that pose health risks, and this method can later be used routinely in testing laboratories.

2. Methodology

2.1. Time and Place of Research

This research was conducted at the Microbiology and Molecular Biology Laboratory, National Food and Drug Testing Development Center, Indonesian FDA. The study was conducted from February to March 2023.

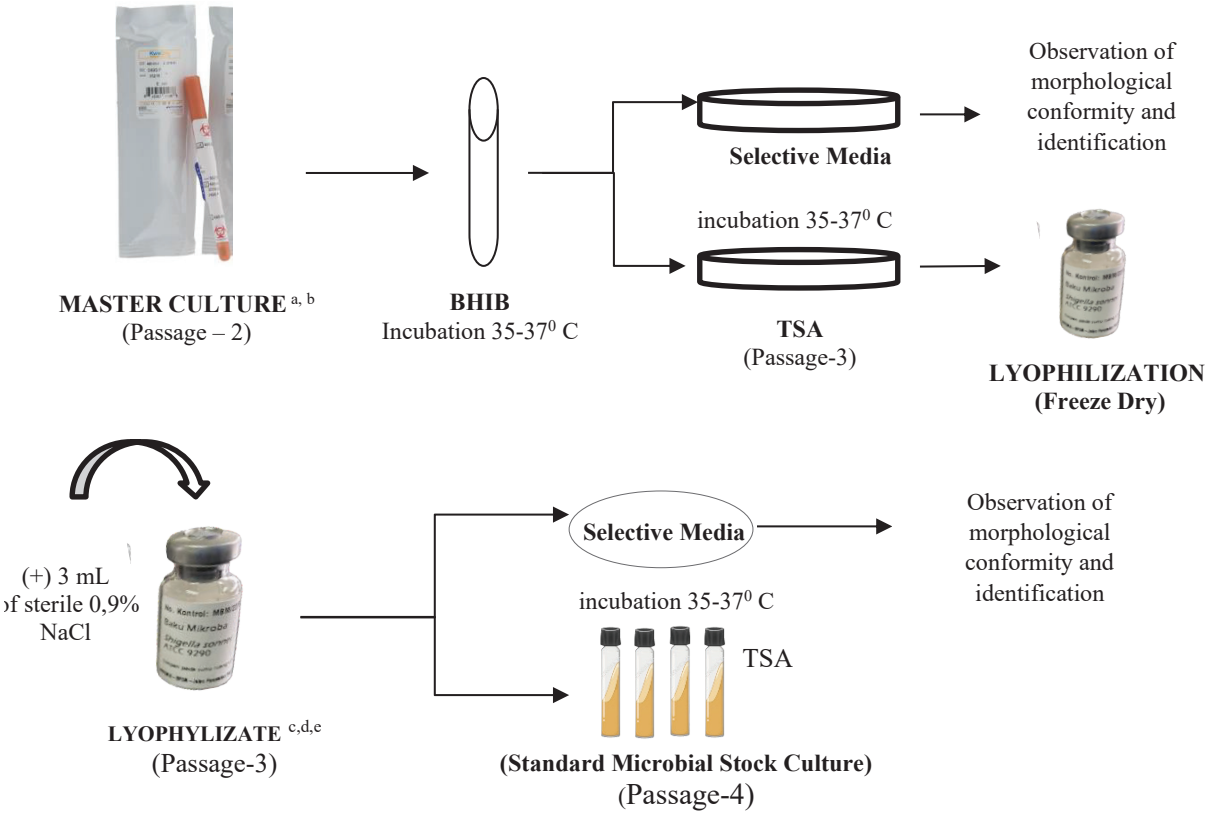
2.2. Materials and Research Instruments

The materials used in this study include Tryptone Soy Broth (TSB), *Enterobacteriaceae* Enrichment Broth Mossel (EEB-Mossel), Mac Conkey Agar (MCA), Xylose Lysine Desoxycholate Agar (XLD), Kligler Iron Agar (KIA), Tryptone Soy Agar (TSA), Buffered Sodium Chloride-Pepton Solution pH 7.0, physiological NaCl solution 0, 9%, Mc Farland standard, GN VITEK® card, quasi oral lozenges that have Indonesian FDA marketing license with the same batch number, raw microbes *Shigella sonnei* ATCC 9290, *Shigella dysentriae* ATCC 13313, *Shigella flexneri* ATCC 9199, *Escherichia coli* ATCC 8739 and *Salmonella typhimurium* ATCC 14028.

The tools used in this study include a set of glassware, oven, autoclave sterilization and deconstruction, incubator, microscope, pH meter, top loading scale, stomacher, Ose needle, Ose incinerator, Biosafety Cabinet (BSC), vortex mixer, micropipette and pipette tips—biochemical identification of bacteria using VITEK® 2 System instrument.

2.3. Preparation of Raw Microbial Cultures

Standard commercially available microbes such as ATCC (American Type Culture Collection) or other collection cultures are used. Standard microbes used for validation should not exceed 5 passages (United States Pharmacopeia Convention, 2022). The standard microbes *Shigella sonnei*, *Salmonella typhimurium* and *Escherichia coli* used in this study used the National Food and Drug Testing Development Center standard microbial lyophilization derived from the master Microbiologics® ATCC pasase-2 culture.



[a] *Shigella dysentriae* ATCC 13313, [b] *Shigella flexneri ATCC 9199*, [c] *Shigella sonnei* ATCC 9290, [d] *Escherichia coli* ATCC 8739, [e] *Salmonella* TyphimuriumATCC 1402

Figure 1. Schematic of Raw Microbial Culture Preparation

The preparation of standard microbial cultures that will be used for validation is illustrated in Figure 1. Indonesian FDA standard microbial lyophilization (pasase-3) was dissolved by adding 0.9% physiological NaCl and then waited for 10-15 minutes. It was then streaked onto several TSA and selective media, such as MCA and XLD, to observe its morphology. Colonies that grow on TSA media (please-4) can be used as raw microbial cultures for spiking.

2.3. Sample Preparation

The samples used were complex candy-type lozenges with Indonesian FDA marketing authorization on the market. The lozenges were aseptically crushed using a sterile mortar and pestle until smooth, then weighed 10 g into a sterile stomacher bag.

2.4. *Shigella* Detection Analysis Using WHO Reference Method

Shigella detection was performed using the reference method from WHO (2011) Quality Control Methods for Herbal Materials listed in Chapter 18: Determination of Microorganisms. The test began with sample preparation, followed by adding 90 mL of TSB as an enrichment medium, then homogenized using a stomacher and incubated at 30-37°C for 2-5 hours (pre-enrichment stage). From the pre-enrichment suspension, 10 mL was pipetted into 90 mL of EEB-Mossel, then incubated at 35-37°C for 18-24 hours. One ose of the enrichment results (EEB-Mossel) was inoculated on the surface of XLD and MCA media and then incubated at 35-37°C for 18-24 hours.

Observations were made of the specific colonies that grew. Cultures were considered *Shigella* positive if there were colonies with the following characteristics:

- On XLD: small round, red, 1-2 mm in diameter.
- On MCA: convex round, colourless, 2-3 mm diameter.

If no colonies grow on MCA and XLD, then *Shigella* is negative. Specific colonies were propagated by inoculating 3 colonies onto TSA/NA slant, incubated at 35-37°C for 24 hours for a confirmation test. The growing colonies were confirmed through Gram staining, KIA/TSIA biochemical media, and automatic bacterial identification using VITEK 2 Compact with GN reagent cards.

The following *Shigella* detection analysis procedure in schematic form is presented in Figure 2.

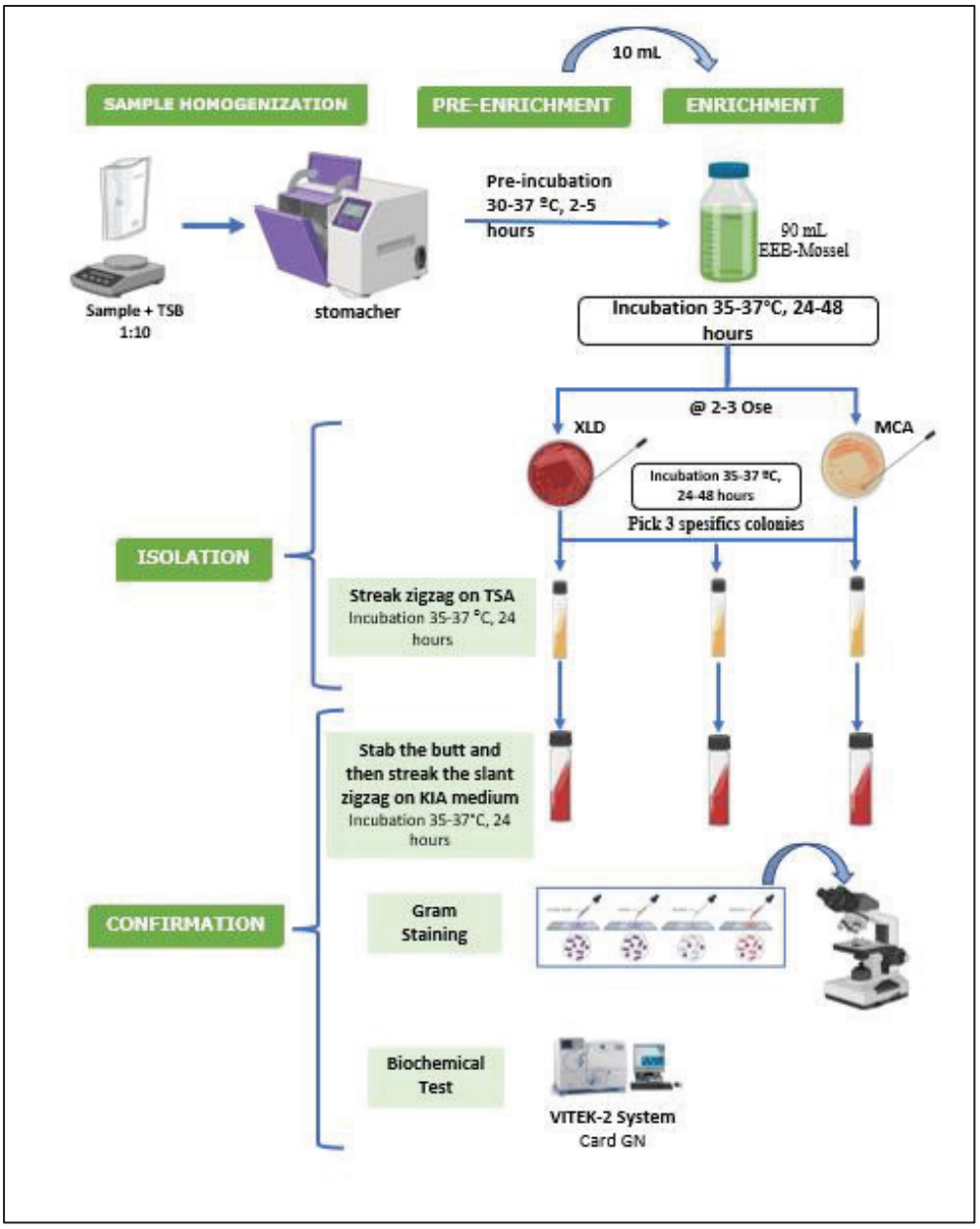


Figure 2. *Shigella* Detection Analysis Test Scheme (World Health Organization, 2011)

2.5. Preparation of contaminant inoculum

Preparation of bacterial suspensions for contamination is based on the calculation of the number of target bacteria (*Shigella sonnei*, *Shigella dysentriae*, *Shigella flexneri*) using the Total Plate Numbers method with 1 McFarland standard or UV-Vis Spectrophotometer (% Transmittance) λ 580 nm. One Ose of standardized microbial culture was inoculated onto TSA media and incubated at 37°C for 24 hours. The growing colonies were suspended in 0.9% physiological NaCl solution until the turbidity reached 1 McFarland. The number of colonies was counted using the Total Plate Count technique. This standardized microbial suspension was inoculated into the sample preparation with a volume according to the required concentration level.

2.6. Method Conformance Test (Optimization)

The suitability test will be conducted for each new product or sample using microbiological methods. This test aims to ensure that the product or sample does not interfere with detecting the analysis results. The test is performed by analyzing negative samples (samples without contamination), positive samples (samples contaminated with *Shigella* sp. standard microbes), and positive controls (solvents without samples contaminated with *Shigella* sp. standard microbes). Positive samples and positive controls are contaminated with a standardized microbial inoculum of no more than 100 cfu and then tested according to the reference analytical method with the shortest incubation period. The positive samples' and controls' test results must be positively detected according to the indicative reaction described in the reference method. The suitability test is declared successful if the positive sample and positive control can grow *Shigella* sp. microbes while the negative sample shows no growth of *Shigella* sp. microbes.

2.7. Measurement or Data Collection

Validation of the *Shigella* test method refers to the microbiology method validation guide (Singapore Accredited Council, 2019). The laboratory must meet acceptance parameters, including 100% sensitivity and specificity and 0% false positive and negative rates. In addition, the method's detection limit (LOD) must be below 100 cfu.

2.7.1. Determination of Limit of Detection

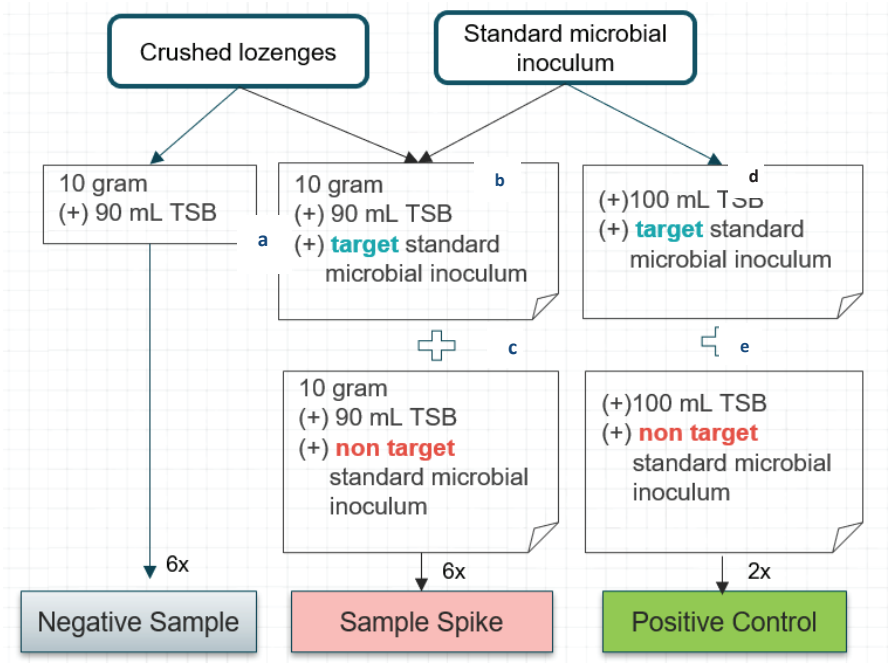
The detection limit was determined by analyzing samples spike at low concentrations of ±1, 3, 9 cfu/g. Each concentration level was tested for 6x replicates using the reference method. The limit of detection was determined by observing the response that gave all positive results from 6x replicates at the slightest concentration. The Singapore Accredited Council validation guidelines, 2019, only mention that it must be detectable below 100 cfu, which means that if the sample weighing is 10 g, the lowest concentration will be below 10 cfu/g. The most minor concentration is 1 cfu/g, while 3 and 9 are multiples of 3.

2.7.2. Determination of Sensitivity, Specificity, False Positive & Negative Rate

Determination of sensitivity, specificity, false positive and false negative rates was carried out according to validation guidelines (Singapore Accredited Council, 2019) by analyzing negative samples, samples spike and positive controls. As shown in Figure 3, the test scheme for sensitivity, specificity, and false positive and false negative rates was carried out by contaminating samples spike and positive controls using microbial inoculum according to the concentration obtained from determining the limit of detection. Samples spike and positive controls were contaminated with three and two non-target microbial strains. Tests were performed using the reference analytical method, with six replicates for samples and two for controls. Negative samples containing only crushed lozenges and diluted with TSB without microbial inoculum were tested six times.

- Negative sample = sample without the addition of any contaminants
- Sample positive spike = sample contaminated with target standard microbes (*Shigella sonnei*, *Shigella dysentriae*, *Shigella flexneri*) and non-target standard microbes (*Escherichia coli*, *Salmonella* Typhimurium)
- Positive control = Solvent media contaminated with target standard microbes (*Shigella sonnei*, *Shigella dysentriae*, *Shigella flexneri*) and non-target standard microbes (*Escherichia coli*, *Salmonella* Typhimurium)

- Negative control = media blank



Description: The difference in treating negative samples, approving samples, and positive controls to determine sensitivity, specificity, and false positive and negative favourable rates lies in the sample homogenization or initial dilution stage.

a. Negative sample = 10 g sample + 90 mL TSB (6x replicates)
b. Target Sample Spike = 10 g sample + 90 mL TSB + target microbial inoculum (*Shigella sonnei*, *Shigella dysentriae*, *Shigella flexneri*) (6x replicates)
c. Non-Target Sample Spike = 10 g sample + 90 mL TSB + non-target microbial inoculum (*Escherichia coli* and *Salmonella* Typhimurium) (6x replicates)
d. Target Positive Control = 100 mL TSB + target microbial inoculum (*Shigella sonnei*, *Shigella dysentriae*, *Shigella flexneri*) (2x replicates)
e. Non-target Positive Control = 100 mL TSB + non-target microbial inoculum (*Escherichia coli* and *Salmonella* Typhimurium) (2x replicates)

Figure 3. Test Scheme of Sensitivity, Specificity, False Positive and Negative Rate

2.8 Data Analysis

Data analysis was performed using descriptive statistical analysis. A descriptive-analytical method is a method that serves to describe or give an overview of the object under study through data or samples that have been collected as is without analyzing and making conclusions that apply to the public (Sugiyono, 2013). Validation categories and calculation formulas refer to Tables 1 and 2 (Singapore Accredited Council, 2019).

Table 1. Validation Categories (Singapore Accredited Council, 2019)

Number of test results	Presumptive (+)	Presumptive (-)	
Confirmed (+)	a	b	a+b
Confirmed (-)	c	d	c+d
	a+c	b+d	n

Description: n = total number of tests
Category a = number of confirmed positive presumptive positives (true positives)
Category b = number of presumptive negatives confirmed positive (false negatives)
Category c = number of presumptive positive confirmed negatives (false positives)
Category d = number of confirmed negative presumptive negatives (true negatives)

After calculating the number of categories a, b, c, and d, input the category using the calculation formula according to Table 2. to determine whether the method validation we have done meets the acceptance requirements.

Table 2. Calculation Formula

Parameter	Calculation Formula
Sensitivity	a/(a+b) x 100%
Specificity	d/(c+d) x 100%
False positive rate	c/(a+c) x 100%
False negative rate	d/(c+d) x 100%

3. Results and Discussion

3.1. Method suitability test (optimization)

The method suitability test is carried out to ensure that no product/sample effect will obscure the analysis results (United States Pharmacopeia Convention, 2022). The results of the method suitability test for *Shigella* detection analysis using TSB solvent media (1:10) with a concentration of 50 cfu/g are presented in Table 3.

Table 1. Method Conformance Test Results

Test Microbe	Inoculum Level	Positive Control	Positive Sample	Negative Sample
Shigella sonnei	50 cfu/g	+	+	-
Shigella flexneri	50 cfu/g	+	+	-
Shigella dysentriae	50 cfu/g	+	+	-

Description:
(+) = there is growth
(-) = no growth

The results of the method suitability test in Table 3. show that all *Shigella* sp. test microbes, namely *Shigella sonnei*, *Shigella flexneri*, and *Shigella dysenteriae* with an inoculum level of 50 cfu/g (below 100 cfu/g), showed growth (+) in the positive control and positive samples. This indicates that the method used can detect the presence of these microbes under the specified conditions.

In the negative sample, there was no microbial growth (-), meaning there was no contamination or unwanted microbial growth under supposedly sterile conditions. This indicates that the test method works well and is reliable in detecting or identifying the tested microbes as intended.

Thus, the analysis of *Shigella* detection in lozenge samples analyzed using the WHO method with the initial solvent TSB (1:10) met the method's suitability requirements without needing prior neutralization/modification procedures for the sample. Microbiological method suitability requirements, as listed in the USP pharmacopoeia guidelines, usually cover several essential aspects, including the ability of the method to detect the growth of target microorganisms under appropriate conditions without interference from external factors such as the physicochemical properties of the sample. In this context, the starting

solvent used was TSB at a ratio of 1:10, a commonly used enrichment medium in microbiology to support the growth of various microorganisms, including *Shigella*.

This method is said to meet the suitability requirements of the technique because:

- Appropriate Growth (Recovery): One of the main criteria of microbiological method validation is the growth of the target microorganism in positive controls and positive samples. In this case, the growth of *Shigella* in both the control and sample indicates that there are no inhibiting factors from the sample that affect the detection results.
- No Neutralization or Modification Required: Usually, in some cases, samples with specific physicochemical properties may inhibit the growth of microorganisms, so neutralization or modification of the procedure is required. However, in this case, the lozenge sample does not require any additional adjustments, which means that the standard method used is optimal for detecting *Shigella*.
- Proven Method Suitability: Validation was performed by ensuring that the method could detect *Shigella* in positive controls (without the influence of the sample) and positive samples (containing both the sample and the target microorganism). The method was qualified without additional modification when consistent growth was observed in both conditions.

Therefore, this validation showed that the WHO method effectively detected *Shigella* in lozenge samples and met the requirements per international standards.

3.2. Limit of Detection (LOD) Determination

A test with a limit of detection was conducted to determine the sensitivity of the test method. The detection limit was determined by analyzing positive samples from various low inoculum concentrations of 1, 3, and 9 cfu per gram. The LOD value was determined based on the lowest concentration of test microbial inoculum in the sample, which could still be detected in all replicates.

Table 2. Detection Limit Determination Test Results

Contaminant Concentration	Number of positive results / Number of tests	Positive Response
Sample + *S.sonnei* 9 cfu/g	6 / 6	100 %
Sample + *S.sonnei* 3 cfu/g	6 / 6	100 %
Sample + *S.sonnei* 1 cfu/g	5 / 6	83,3 %

Based on Table 4, samples contaminated with *Shigella sonnei* at concentrations of 9 and 3 cfu/g showed positive results in all 6 test samples (100%). However, at a concentration of 1 cfu/g, only 5 out of 6 test samples (83.3%) gave positive results, while 1 other sample did not detect the presence of *Shigella* sp.

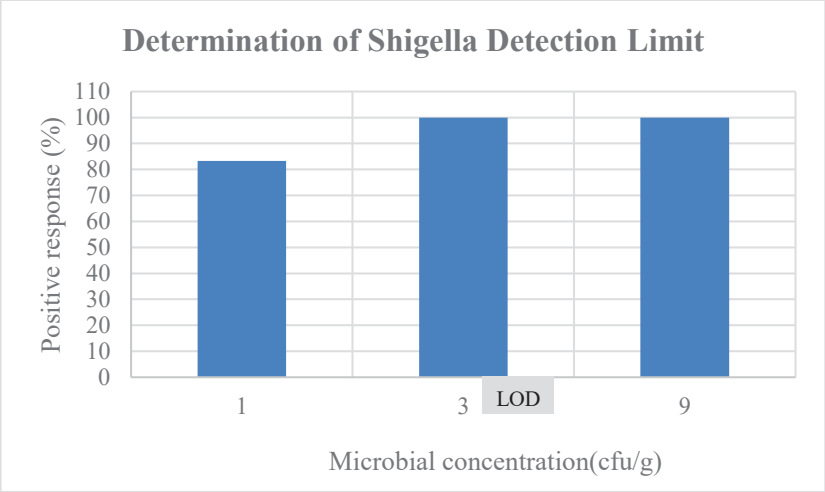


Figure 1. Determination of Limit of Detection

Based on the detection limit concentration determination test (Figure 4), the LOD value of the *Shigella* spp. The detection method was obtained at 3 cfu/g sample. This concentration is the lowest, giving 100% of the test microbes detected. According to the Indonesian Pharmacopeia (2020), microbial detection methods must be able to detect microbes with numbers below 100 colonies per gram of sample. Still, the actual detection limit has never been determined quantitatively because many variables can affect microbial recovery in preparations. The results of the limit of detection determination can be used as a reference for the LOD value for contaminant inoculum concentration in the validation and verification of an analytical method.

3.3.Method Validation Results

Data from the sensitivity, specificity, false positive rate and false negative rate tests on the validation of the *Shigella* detection analysis method are presented in Table 5 and the sum of the results in Table 6.

Table 3. Method Validation Testing Results

Analysis	Repeat	Presumptive	Confirmation	Category
Negative Sample	1	Negative	Negative	d
	2	Negative	Negative	d
	3	Negative	Negative	d
	4	Negative	Negative	d
	5	Negative	Negative	d
	6	Negative	Negative	d
Positive Sample *S.sonnei* (target microbe)	1	Positive	Positive	a
	2	Positive	Positive	a
	3	Positive	Positive	a
	4	Positive	Positive	a
	5	Positive	Positive	a
	6	Positive	Positive	a
Positive Control *S.sonnei* (target microbe)	1	Positive	Positive	a
	2	Positive	Positive	

Analysis	Repeat	Presumptive	Confirmation	Category
				a
Positive Sample *S.flexneri* (target microbe)	1	Positive	Positive	a
	2	Positive	Positive	a
	3	Positive	Positive	a
	4	Positive	Positive	a
	5	Positive	Positive	a
	6	Positive	Positive	a
Positive Control *S. flexneri* (target microbe)	1	Positive	Positive	a
	2	Positive	Positive	a
Positive Sample *S.dysentriae* (target microbe)	1	Positive	Positive	a
	2	Positive	Positive	a
	3	Positive	Positive	a
	4	Positive	Positive	a
	5	Positive	Positive	a
	6	Positive	Positive	a
Positive Control *S.dysentriae* (target microbe)	1	Positive	Positive	a
	2	Positive	Positive	a
Positive Sample *E.coli* (non-target microbes)	1	Negative	Negative	d
	2	Negative	Negative	d
	3	Negative	Negative	d
	4	Negative	Negative	d
	5	Negative	Negative	d
	6	Negative	Negative	d
Positive Control *E.coli* (non-target microbe)	1	Negative	Negative	d
	2	Negative	Negative	d
Positive Sample *S.*Typhimurium (non-target microbe)	1	Negative	Negative	d
	2	Negative	Negative	d
	3	Negative	Negative	d
	4	Negative	Negative	d
	5	Negative	Negative	d
	6	Negative	Negative	d
Positive Control *S.*Typhimurium (non-target microbe)	1	Negative	Negative	d
	2	Negative	Negative	d

Based on Table 5, the presumptive determination of *Shigella* sp. is as follows:

- In negative samples, the presumptive result is negative.
- The presumptive result was positive in positive samples and positive controls contaminated with target microbes (*Shigella sonnei*, *Shigella flexneri* and *Shigella dysentriae*).
- The presumptive results were negative in positive samples and positive controls contaminated with non-target microbes (*E. coli* and *Salmonella* Typhimurium).

Negative samples did not show colony growth, so the confirmation result was negative. In contrast, a complete confirmation test (MCH scratch stain, Gram stain, and automatic identification with VITEK 2) was performed to approve samples and positive controls and

determine whether the colonies growing on MCA and XLD selective media were positive or negative.

After obtaining the presumptive-confirmation test results from each treatment, the results were grouped as follows: Negative presumptive results that were confirmed negative were categorized as "d". Positive presumptive results that were confirmed positive were classified as "a". No test results fell into categories "b" or "c".

Table 4. Number of Validation Test Result Categories

Number of test results	Presumptive (+)	Presumptive (-)	
Confirmed (+)	a = 24	b = 0	24
Confirmed (-)	c = 0	d = 22	22
	24	22	n = 46

After grouping and categorizing the test results, as shown in Table 6. there are 24 for Category A and 22 for Category D, with 46 tests.

Tabel 5. Calculation of Sensitivity, Specificity, False Positive and Negative Rates

Parameter Validation	Calculation	Conclusion
Sensitivity	$\frac{a}{a+b} = \frac{24}{24+0} = 100\ \%$	Qualified
Qualified	$\frac{d}{c+d} = \frac{22}{0+22} = 100\ \%$	Qualified
False Positive Rate	$\frac{c}{a+c} = \frac{0}{24+0} = 0\ \%$	Qualified
False Negative Rate	$\frac{b}{b+d} = \frac{0}{0+22} = 0\ \%$	Qualified

In the calculation based on Table 7. the sensitivity and specificity values obtained the results of 100%. These results indicate that the method used meets the requirements for detecting *Shigella* sp. microbes, specifically in the presence of other components in the sample matrix. According to the Singapore Accredited Council (2019), the difference between sensitivity and specificity is that sensitivity is the ability of the test method to give positive results on samples that have been contaminated with target microbes (*Shigella sonnei*, *Shigella flexneri* and *Shigella dysentriae*) while specificity is the ability of the test method to give negative results on samples that do not contain target microbes or samples contaminated with non-target microbes (*Escherichia coli* and *Salmonella typhimurium*).

The test results show that the false positive and false negative rate is 0%. All samples that should be positive gave positive results, and all samples that should be negative gave negative results. Thus, the *Shigella* spp. The detection method tested using the WHO method is reliable. The false positive rate test was performed to determine the possibility of a negative sample giving a positive result due to the influence of reagents, media used, or contamination due to non-aseptic procedures. Conversely, a false negative test is performed to determine the possibility of a positive sample giving a negative result due to the influence of the sample matrix or reagent (Ismail, 2009).

This method qualifies validation parameters, including sensitivity, specificity, and false positive and negative rates (World Health Organization, 2011). Therefore, the WHO method

is very applicable in testing laboratories throughout the POM Agency. With a sensitivity result of 100% and a detection limit of 3 cfu/g, this method allows for routine analysis.

The WHO analytical method (Quality control methods for herbal materials) for the detection of *Shigella* in quasi-medicinal lozenges that have been validated in this study can be proposed as an analytical method text (MA-PPPOMN) and formalized in the MA-PPPOMN discussion session. This method can then be officially used in all Indonesian FDA laboratories in Indonesia to monitor *Shigella* microbial contamination in quasi-medicines

4. Conclusion

This study aims to validate the method used for *Shigella* detection analysis in quasi-medicinal lozenges using the WHO reference method (Quality control methods for herbal materials). The results showed that the technique had a sensitivity and specificity of 100% and a false positive and false negative rate of 0%. The limit of detection obtained was 3 cfu/g, which meets the acceptability requirements of microbiological analysis methods (below 100 cfu/g). With these validation results, the process is declared accurate and can be applied for routine *Shigella* spp—testing analysis in the Food and Drug Administration laboratories.

Acknowledgements

The authors would like to thank the leadership and staff of the National Food and Drug Testing Development Center, Indonesian FDA, for all the contributions made so that this research can be carried out correctly. Our gratitude also goes to Mr Decky Indrawan Junaedi, M.Env., Ph.D., who is the supervisor of this journal writing.

References

Aini, N., Purba, M., Triwibowo, S., Sholihatul, N., & Wulandari, R. (2021). Penetapan Limit of Detection (LOD) dan Sensitivitas Metode Deteksi Pseudomonas aeruginosa dalam Berbagai Matriks Sediaan Obat. *Eruditio : Indonesia Journal of Food and Drug Safety*, *1*(2). https://doi.org/10.54384/eruditio.v1i2.85

Brooks, G. F., Carroll, K. C., Butel, J. S., Morse, S. A., & Mietzner, T. A. (2012). *Jawetz Melnick & Adelberg's Medical Microbiology* (26th ed.). McGraw-Hill LANGE.

Choursiya, S., & Andheriya, D. (2018). Review on Lozenges. *Journal of Drug Delivery and Therapeutics*, *8*(A), 124–128. http://jddtonline.info

Faridah, D. N., Erawan, D., Sutriah, K., Hadi, A., & Budiantari, F. (2018). *Implementasi SNI ISO/IEC 17025:2017 Persyaratan Umum Kompetensi Laboratorium Pengujian dan Laboratorium Kalibrasi*. Badan Standardisasi Nasional.

https://cekbpom.pom.go.id/obat_kuasi (diakses tanggal 24 Oktober 2023). (n.d.).

Korir, R., Anzala, O., Jaoko, W., Bii, C., & Keter, L. (2017). Multidrug-Resistant Bacterial Isolates Recovered from Herbal Medicinal Products Sold in Nairobi, Kenya. *East African Health Research Journal*, *1*(1). https://doi.org/10.24248/eahrj.v1i1.386

Kotloff, K. L., Riddle, M. S., Platts-Mills, J. A., Pavlinac, P., & Zaidi, A. K. M. (2018). Shigellosis. In *The Lancet* (Vol. 391, Issue 10122). https://doi.org/10.1016/S0140-6736(17)33296-8

Liew, P. S., Teh, C. S. J., Lau, Y. L., & Thong, K. L. (2014). A real-time loop-mediated isothermal amplification assay for rapid detection of *Shigella* species. In *Tropical Biomedicine* (Vol. 31, Issue 4). http://blast.ncbi.nlm.nih.gov/Blast.cgi

Muzembo, B. A., Kitahara, K., Mitra, D., Ohno, A., Khatiwada, J., Dutta, S., & Miyoshi, S. I. (2023). Burden of *Shigella* in South Asia: a systematic review and meta-analysis. In *Journal of Travel Medicine* (Vol. 30, Issue 1). https://doi.org/10.1093/jtm/taac132

Peraturan BPOM No.7 Tahun 2023 Tentang Kriteria Dan Tata Laksana Registrasi Obat Kuasi (2023).

Singapore Acredited Council. (2019). *Acreditation Scheme for Laboratories. Guidance Notes C&B AND ENV 002 Method Validation of Microbiological Methods*. The SAC Accreditation Programme by SPRING Singapore.

United States Pharmacopeia Convention. (2022). *USP-NF <62> Microbiological Examination of Nonsterile Products: Tests For Specified Microorganisms*. https://doi.org/10.31003/USPNF_M98802_01_01

Walusansa, A., Asiimwe, S., Kafeero, H. M., Stanley, I. J., Ssenku, J. E., Nakavuma, J. L., & Kakudidi, E. K. (2021). Prevalence and dynamics of clinically significant bacterial contaminants in herbal medicines sold in East Africa from 2000 to 2020: a systematic review and meta-analysis. *Tropical Medicine and Health*, *49*(1). https://doi.org/10.1186/s41182-020-00295-8

World Health Organization. (2011). *Quality control methods for herbal materials.* World Health Organization.

Yang, J., Zhang, N., Lv, J., Zhu, P., Pan, X., Hu, J., Wu, W., Li, S., & Li, H. (2020). Comparing the performance of conventional PCR, RTQ-PCR, and droplet digital PCR assays in detection of *Shigella*. *Molecular and Cellular Probes*, *51*. https://doi.org/10.1016/j.mcp.2020.101531