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# EMPOWERING FUTURE HEALTHCARE PROFESSIONALS: A COMPREHENSIVE APPROACH TO MICROBIOLOGY TESTING FOR VOCATIONAL STUDENTS

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**ABSTRACT**

Vocational high schools (SMK) are formal educational institutions that play a crucial role in preparing students to be better prepared to face future challenges. Specific training or skills programs in certain fields, such as health, can be provided to students as provisions before entering the workforce. By providing a deeper understanding of microbiology testing from an early age, health vocational school graduates can be better prepared to face industry demands and actively improve safety standards in the health care sector. The safety and effectiveness of medical devices are highly dependent on microbiological control. The curriculum provides students with critical skills and insights related to microbiological testing on various medical devices. The manufacturing process of medical devices, especially Class II and III devices, requires a high level of sterility since they interact with blood, vessels, or internal organs of the human body. Therefore, microbiological testing is essential to ensure sterility. This review analyzes various microbiological testing methods used to ensure the safety of medical devices, focusing on Sterility Testing, Bioburden Testing, and Endotoxin Testing. The purpose of this review article is to provide information on microbiological tests applicable to sterile product evaluation. The method used in this study involves a literature review of Indonesian National Standards (SNI), Indonesian Pharmacopoeia (FI), and international journals. Various studies indicate that sterility testing is performed to ensure that medical device products are free from microbial contamination after sterilization treatment with ethylene oxide (EO) machines. Meanwhile, Bioburden Testing is conducted to determine the bacterial load on Medical Devices before sterilization with EO machines. Endotoxin Testing aims to ensure that Medical Devices are free from bacteria, particularly Gram-negative bacteria, which are pyrogenic and produce toxins in the body.

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## Introduction

Vocational high schools (SMK) are formal educational institutions that play a crucial role in preparing students to be better prepared to face future challenges. Specific training or skills programs in certain fields, such as health, can be provided to students as provisions before entering the workforce. In health vocational schools, inserting and designing microbiology

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materials in the curriculum can equip students to be more prepared, critical, and professional before entering the workforce in the health sector. By providing a deeper understanding of microbiology testing from an early age, health vocational school graduates can be better prepared to face industry demands and actively improve safety standards in the health care sector. The safety and effectiveness of medical devices are highly dependent on microbiological control (Fidiastuti et al., 2024).The curriculum provides students with critical skills and insights related to microbiological testing on various medical devices.

The safety and effectiveness of medical devices heavily depend on microbial control. Microbiological testing of medical devices involves assessing the presence and risk of microbial contaminants. The methods used may include bioburden testing, endotoxin detection, and sterility assurance techniques. These tests are crucial components of quality control and safety measures, as they evaluate the biological risks of contaminants to eliminate or reduce the likelihood of infections in patients. Additionally, these tests are supported by environmental monitoring and microbial control throughout the entire production process. Microbiological testing can also be applied to non-sterile medical devices, as these devices may require monitoring and/or control to detect the presence of unwanted microorganisms (Degen, 2024).

Sterility assurance in medical equipment is one of the requirements established by global medical device regulations. Medical device providers must ensure that the instruments are free from harmful microorganisms. Laboratory medical professionals or medical device facilitators must consistently perform microbiological testing throughout the production process to maintain product quality and ensure patient safety (Kementerian Kesehatan Republik Indonesia Direktorat Jenderal Kefarmasian dan Alat Kesehatan, 2020). Therefore, sterility, bioburden, and endotoxin testing are crucial in ensuring that medical devices used in healthcare environments are safe for patients.

Sterility testing aims to verify the product is completely sterile and free from living microorganisms. The sterilization process is carried out using various methods, including physical techniques (such as autoclaving) and chemical techniques (such as ethylene oxide gas sterilization) with ethylene oxide (EO) sterilization machines. However, evaluating the effectiveness of these methods poses challenges, especially for complex and multi-component medical devices (Setiawati & Lolo Lukita, 2022).

Meanwhile, bioburden testing is conducted to determine the bacterial load present in a product before undergoing sterilization in EO machines. A high bioburden level may indicate potential sterilization failure, directly impacting patient safety. Identifying and monitoring bioburden levels are vital in supporting appropriate preventive measures. Bioburden and sterility testing are performed together to monitor microbial presence (Setiawati & Lolo Lukita, 2022)

On the other hand, endotoxin testing, particularly for Gram-negative bacteria, is a critical aspect of microbial testing to measure the presence of pyrogenic substances (fever-inducing agents) derived from bacterial endotoxins. Endotoxins can trigger severe reactions in the human body, such as septic shock. Therefore, medical devices must be tested to ensure that endotoxin levels remain below the established safety thresholds. The Limulus Amebocyte Lysate (LAL) method is commonly used for this testing (Schlottrerer & Wolff, 1996).

In this review, the author will further analyze the importance of considering these factors, emphasizing the need for an in-depth study of bioburden testing, endotoxin testing, and sterility testing methods. This is essential to develop efficient and effective testing protocols and ensure that all marketed medical devices comply with established safety and quality standards. These efforts will not only enhance public trust in medical products but also safeguard overall patient health.

## Methods

This review article was compiled using a literature search study technique. Sources of information related to the theoretical review of sterility testing, bioburden assessment, and endotoxin detection were obtained from:

1. Guidelines issued by international and national institutions/organizations (such as the Indonesian Food and Drug Authority) accessed through their official websites, including jdih.pom.go.id, picscheme.org, www.tga.gov.au, www.fda.gov, and www.who.int.
2. Guidelines in the form of the Indonesian Pharmacopoeia (FI), Indonesian National Standard (SNI), British Pharmacopoeia (BP), European Pharmacopoeia, and United States Pharmacopeia (USP).
3. Collection of information from published journals or articles using search engines such as Google and Google Scholar with the keyword "*sterility testing failure*".
4. Direct acquisition of information regarding the regionalization of sterility laboratories that are currently under discussion.

## Results

### Sterility Test

### A. General Description

Sterility test is a method test of sterile pharmaceutical product or medical product with sterile labeled. Sterile condition can ensure the safety of invasive or non-invasive medical device (Rutala et al., 2023). Medical device such as syringe, infusion fluids, HD solution fluids, heart ring, catheter, and other medical devices require sterile status confirmation before injected into the veins or human body. However, this test remains weak for example a long incubation time and depend on the probability to detect contamination in the homogenous product. The sterility test is highly important due to the regulation. The test frequently showed 'not sterile' result that must be re-test to discover the source of the unsterile cause. The sterility test must be conducted in well-controlled facility (Setiawati & Lolo Lukita, 2022).

### B. Sterility test standard for medical device

In Indonesia, sterility test must be according to Farmakope Indonesia VI Edition (Kementerian Kesehatan Republik Indonesia Direktorat Jenderal Kefarmasian dan Alat Kesehatan, 2020) and test facility requirements in 'Petunjuk Operasional Cara Pembuatan Obat yang Baik Tahun 2012' (BPOM, 2012). The sterility test must be high controlled in accordance to CFR Quality System Requirement, Good Laboratory Practice, environment (clean room ISO class 5 or higher), and competent analyst. The sterility test is a necessary element in validation process of sterilization and quality product control.

The expected result after the sterility test in the EO machine is a sterilized product free from microbial contamination, including bacteria and fungi. The test is conducted in the final process before the product is commercialized. Even though the sterility test cannot guarantee sterility for all batches, it can identify unsterile products within a batch. As a result of aseptic checking using microbiological testing methods, this process can reduce the contamination level in medical devices intended for patient use and help identify the root cause of contamination in sterilized products (Certified Laboratories, n.d.). The required sample size is specified in Farmakope Indonesia and the international guidelines below:

| Number of containers | Minimum number of containers will be tested for each media (except otherwise) |
|---|---|

| | | |
|---|---|---|
| Stock of parenteral | Less than 100 containers | 10% or 4 containers, take the bigger one choose the bigger one |
| | 100 – 500 containers | 10 containers |
| | More than 500 containers | 2% or 20 containers, take the smaller one choose the smaller one |
| | For big stock | 2% or 10 containers take the smaller one, choose the smaller one |
| Antibiotics solid | Packed sterile product less than 5 gram | 20 containers |
| | Packed sterile product more than 5 gram | 6 containers |
| | Processed and mixed products | See the solid processed product |
| Eye drops / supplies and other non-injectable supplies | Less than 200 containers | 5% or 2 containers, choose the bigger one |
| | More than 200 containers | 10 containers |
| | If the stock / supplies are in single-dose container form, use the scheme above for parenteral stock / supplies | |
| | Surgical suture and other surgical device for veterinary use | 2% or 5 packs, choose the bigger one, up to a maximum total of 20 packs |
| | Less than 100 material | 10% or 4 material, choose the bigger one |
| | More than 100, up to 500 material | 10 material |
| | More than 500 material | 2% or 20 material, choose the smaller one |
| | Processed material product | |
| | Up to 4 containers | Each container |
| | More than 4 containers, up to 50 containers | 20% or 4 containers, choose the bigger one |
| | More than 50 containers | 2% atau 10 containers, choose the bigger one |

(Kementerian Kesehatan Republik Indonesia Direktorat Jenderal Kefarmasian dan Alat Kesehatan, 2020)

## C. Sterility test method

In the process of sterility test, the bacteria prevention must be concerned using test in aseptic condition. The room test or laboratory also must be maintenances periodical with proper control. The compounding of sterile preparations is a critical stage that demands careful attention to ensure the quality of the final product remains unaffected (Genatrika et al., 2021). The method of sterility test also has to follow the Farmakope. These are two type of sterility test, membrane filtered method and direct inoculation. Filtered membrane method preferred for product that can be filtered to lowering the risk of false negative result. In this test, the media for cultivation such as Fluid Thioglyocollate Medium is used for anaerob bacteria, while Soybean–Casein Digest is used for anaerob bacteria and fungi. The sterility test aims to confirm the sterility status of the sterile medical device product completely free from bacteria contamination (Iffah et al., 2025).

In the process of media making, the media must be ensured with *Growth Promotion Test (*GPT) and *Method Suitability Test*. GPT has a function to confirm that culture media for sterility test is in a good condition for specific microorganism cultivation if they are present. It focusses on confirmation of media condition appropriateness, preventing media *contamination* chance (Degen, 2024). *Method Suitability Test* aims to proving the sterility test method (including sample preparation and media test) can detect the presence of microorganism in the tested product (Degen, 2024).

Sterility test must be tested using suitability test aiming to ensure that sterility test is conducted in accordance with relevant standard and proper. The result of the suitability test affecting the sterility test. If the suitability test fails and do not match the requirement of the standard the sterility test result is not valid (Iffah et al., 2025).

The test method in the sterility test must be in accordance with the method contained in the pharmacopoeia used. The sterility test method on medical devices consists of 2 methods, namely:

a. Membrane Filtration for filterable products

This method carries out using a sterile membrane to filter microorganisms with a membrane density 0.45 microns. This membrane filtration method is ideal for complex raw materials, such as oily, particulate-rich, or viscous products. In addition, this method is most suitable for large-volume medical device samples or bulk solutions. In carrying out this procedure, a sample in the appropriate amount is required, usually 100-200 mL which will be filtered with membrane filtration. The membrane that filters the sample is cut and then inoculated into two growth media for bacteria and fungi, the two-growth media are Tryptic Soy Broth (TSB) for aerobic organisms and Fluid Thioglycollate Medium (FTM) for anaerobic organisms (Suriawati et al., 2021). Then the media and membrane filter are incubated for 14 days at a temperature of 30-35°C for bacterial growth and 20°-25°C for fungal growth on the inoculated media.

This test is a more preferred test compared to the direct inoculation test, because it has higher accuracy due to the small size of the membrane filter, can be used for large scales and volumes, is more sensitive, and is not easily disturbed by the growth of other bacteria or contaminants. However, because the results of higher accuracy and precision, the method prepared is also more complicated, especially in the process of assembling the filtration equipment used. Membrane filtration requires additional steps, including filtration and membrane removal which makes the process more time-consuming and laborious. As well as the risk of clogging of the membrane used, making the filtration process difficult and requiring a sterile guarantee on the filtration equipment that will be used in the sample filtration process. After the incubation period, data analysis is conducted using qualitative method, by looking at the presence or absence of microorganism growth in the media. Growth is seen as colonies that appear on the incubated media, any microbial contamination trapped in the membrane will grow in the medium, resulting in turbidity in the medium. The results obtained are then compared with positive and negative controls to ensure that the test results are valid (Degen, 2024).

b. Direct Plating for non-filterable products

The direct inoculation method is an easy and practical method to do, where the test sample is directly inserted (or inoculated) into the growth medium, which is then incubated under certain conditions to detect microbial growth. The growth medium used in this method is designed to support the growth of bacteria and fungi. In its use, this method is more suitable for small sample sizes or products that are not compatible with filtration such as medical device products. According to the Indonesian Pharmacopoeia (2020), medical device products in solid form such as syringes, catheters, infusion tubes and other medical equipment in solid form are usually subjected to the sterility test method by direct inoculation.

In carrying out the direct inoculation method, Fluid Thioglycollate and Tryptic Soy Broth (TSB) media can be used. The samples are inoculated into the media or inserted directly into the test media and incubated for 14 days, then observations are made for the presence or absence of microorganism growth in the media. The results of the observed data analysis are in

qualitative form by looking at the turbidity or level of turbidity or clarity in the media which indicates the growth of microorganisms (Degen, 2024).

Observations of turbidity of the media not only on the 14th day after cultivation, but also on days 3, 5, 7, and 14. Reading of the incubation results observed by visually manually. However, this process can be a weakness of the sterility test, the probability of error in the manual visual inspection. If the test results are presented cloudy, visual observation can be confusing, so validation or repeat testing is needed to ensure contamination. Any sign of turbidity triggers subculture and increases the risk of laboratory contaminants entering (Gebo & Lau, 2020). In conducting sterility test methods, both membrane filtration methods and direct inoculation methods, both have similarities in the use of media for incubation in samples. The following are the media used in both sterility test methods:

Fluid Thioglycollate

Fluid Thioglycollate media is a commonly used media in the growth of anaerobic bacteria in sterility tests, this media is generally incubated at a temperature of 30°–35°C. However, there are exceptions to the incubation temperature if the product to be tested on this media contains preservatives such as mercury, usually the media is good to be incubated at a temperature range of 20°–25°C. The pH of Fluid Thioglycollate media after the sterilization process in an autoclave at a temperature of 1210C, a pressure of 1.5 atm for 15 minutes is 7.1±0.2 (Kementerian Kesehatan Republik Indonesia Direktorat Jenderal Kefarmasian dan Alat Kesehatan, 2020).

- Tryptic Soy Broth (TSB)

Tryptic Soy Broth (TSB) media is a media used in the growth of aerobic bacteria in sterility tests. However, in general, Tryptic Soy Broth (TSB) also contains sufficient nutrients for some types of fungi or yeast, especially those that are dimorphic or yeast fungi (such as *Candida* spp.). However, this media does not contain high levels of dextrose like those in SDA or PDA. Based on the Indonesian Pharmacopoeia, this media can be used for sterility testing, both membrane filter methods and direct inoculation methods, which in testing solid Medical Devices require liquid media in the process. However, unlike Fluid Thioglycollate media, the pH of this media after sterilization is 7.3 ± 0.2 and incubated at a temperature of 22.5 ± 2.5 ° C (Kementerian Kesehatan Republik Indonesia Direktorat Jenderal Kefarmasian dan Alat Kesehatan, 2020).

**Bioburden Test**

**A. General Description**

Bioburden can be defined as the number of bacteria who living on a surface that has not been sterilized. While the bioburden test is on type of microbiological test that is often known as the total viable count test (TVC). This microbiological test has a purpose of estimating the presence of aerobic mesophilic microorganisms in a product or item with a status that is declared non-sterile (Clontz, 2008). However, in the context of microbiology, the term of bioburden is also often defined as a biological burden which sometimes implies another meaning that the presence of microorganisms will be a problem or concern. Though in practice, the purpose of this bioburden test is to confirm what type and number of microorganisms present on an object is suitable when compared to the predetermined acceptance criteria (Hodges, 2004).

In some literature, bioburden testing is also related to raw material testing, environmental area monitoring, or sample testing. All three areas have a natural biological burden and when this natural biological burden increases above a predetermined level, the correct term to call this phenomena is biocontamination. This biocontamination phenomena

will be a problem when found on an object or environmental area because of the explosion of microorganism populations which will be considered a serious problem if found in the pharmaceutical context or medical device production (Sandle, 2016).

**B. Bioburden test standard for medical devices**

In the context of medical device production, bioburden assessment is a primary requirement that must be completed before going to another stage or sterility test. This is because the purpose of the bioburden analysis itself is to monitor the total number of microbes that can live in or on a medical device so that it can affecr the quality of the product and can also affect its stability (Gold & Phelps, 2017).

Natural biological burden testing for terminally sterilized medical devices or pharmaceutical products has the purpose of comfirming that the pre-sterilization natural biological burden is under the specified limit or is used to qualify the sterility test process. The limitations and conditions of bioburden testing for medical devices refer to ISO 11737 that is used worldwide (Sandle, 2016). ISO 11737 is a standard that specifically regulates and discusses the determination of viable populations of microorganisms on or in medical devices, components, raw materials or packaging (Schlottrerer & Wolff, 1996).

The control of bioburden testing on medical devices, as explained in the ISO 11737 standard, consist of the three main steps as follows:

1. Selection and collection process of microorganism from medical device samples
2. Transfer of microorganisms to the recovery solution
3. Counting or enumeration of collection samples containing recovered microorganism (enumeration)

**C. Bioburden testing methods**

**a. Sample selection and microorganism collection process**

According to the USP, all medical device samples for bioburden testing should be prepared or collected using appropriate methods to maintain the stability of the natural biological burden in the sample. If the medical device sample contains a high natural biological burden, it should be diluted first to obtain a more homogenous sample (Clontz, 2008). All stages of preparation are conducted using aseptic techniques, including disinfecting the work area, using LAF, using sterile materials, and utilizing fire to prevent unwanted contamination from pathogenic microorganism (Sandle, 2016).

In addition, laboratory staff must also maintain hygiene, avoid direct contact with instrument that come into contact with samples, and understand all types of medical device products to be tested (Sandle, 2016). However, almost all medical device products to be tested through bioburden testing usually come from solid products (e.g., plastics and metals). These products then go through an extraction step using a water-based or sterile NaCl solution followed by testing all or part of the extraction solution using membrane filtration, pour plate, or spread plate (Filaire et al., 2023).

According to ISO 11737-1, for medical device samples to be extracted, the sample extraction treatment should be in accordance with the following product type classification:

- ***Non-flexible Solid Product***

  For samples derived from non-flexible solid products, the first step is to rinse or soak the sample with sterile 0,7% NaCl solution and collect the liquid in a sterile bottle then extract using a vortex or shaker.
- ***Products with Foam and or Fiber***

For samples derived from foan or fiber-based products, the first step is to put the sample directly into a bottle containing sterile 0,7% NaCl and then extract it with a vortex or shaker.

- ***Product with lumen***
  For product samples with a lumen, the first step is to rinse the product with sterile 0,7% NaCl using a syringe by flowing over the entire lumen and collecting the liquid in a sterile bottle. Or it can be done by cutting the product into several small pieces and then putting them in a bottle containing sterile 0,7% NaCl liquid. Then extract using a vortex or shaker.
- ***Semi-solid or Powdered Products***
  For semi-solid or powdered product samples, the first step is to put the sample directly into a bottle containing sterile 0,7% NaCl. Then extract using a vortex or shaker. The dilution of microorganism cell suspensions should not carried out in an unfavorable environment. Diluted microorganism cells are more susceptible to damage than denser concentrations of cells. The use of sterile NaCl solution as a buffer solution at the stage of collecting microorganisms in medical device products for bioburden testing has the purpose of diluting microorganisms and protecting cells from pH changes after cells are suspended (Filaire et al., 2023).

**b. Removal and Calculation of Samples After Extraction**

After completing the extraction stage to collect microorganism cells from the medical device product stock into a suspension solution, bioburden testing will then be carried out using several methods specified in the standardization. To examine or count the number of microorganism, there are four recommended methods to follow such as: membrane filtration method, pour plate method, and spread plate method (Sandle, 2016).

But before that, it is required to prepare and know about the type of growth media and incubation conditions that depend on the type of microorganism to be counted. Selective media or differential media can be used to show the growth of certain types of microorganism in order to obtain specific results (Filaire et al., 2023). According to (Peng, 2020), microorganism growth media have a vital role to show that the media supports growth and has the ability to detect organisms from test samples. There are two types of growth media can be used, the first is *Tryptic Soy Agar* (TSA) which is used for bacterial cultures such as *Staphylococcus aureus*, *Pseudomonas aeruginosa,* and *Bacillus subtilis*. As for the second growth medium, *Sabouraud Dextrose Agar* (SDA) is used for *yeast* cultures such as *Candida albicans* and *Aspergillus niger* (Acharya & Hare, 2022). For the cultivation of test microorganism, the growth medium must contain an inactivator (neutralizer) suitable for the spesific antimicrobial properties in the growth medium used for the test procedure (Sandle, 2016).

After determining the type of microorganism growth media that will be used in the bioburden test, the next step is to calculate the number of microorganism using the four methods that previously mentioned, as shown below:

**i. Membrane Filtration Method**

With this method, microorganism samples are filtered through membrane filters with a pore size of 0.45μm or less. After passing through the filtration stage, each filter should be washed three or more times with suitable solution such as phosphate buffer, sodium chloride-peptone buffer or other recommended buffer with a washing volume that should reach about 100 mL. For bacteria detection, both filters should be placed on top of a petri dish containing TSA medium. And for *yeast* detection, antibiotics are added to the media and placed on top of a petri dish of one the SDA medium. Petri dishes containing agar media and inoculated microorganism are incubated for at least 5 days at 30-35°C for bacterial detection and at 20-

25°C for *yeast* detection. At the termination of the incubation period, the number of colonies was counted.

**ii. Pour Plate Method**

Using the pour plate method, each dilution must use two agar media which are put into a petri dish with a diameter of 9-10 cm. Take 1 mL of test sample suspension and put it into each petri dish aseptically, then add 15-20 mL of sterilized agar medium that has been previously stored under 45°C and then mix it. Temperatures at or above 45°C can cause heat-induced cell death. For bacteria detection, use TSA medium and for *yeast* detection use SDA medium to which antibiotics have been previously added. After the agar medium stiffens, incubate for approximately 5 days at 30-35°C for bacterial detection and at 20-25°C for *yeast* detection. After a large number of colonies have developed, count the number of viable colonies with no more that 300 colonies/dish for bacteria detection and no more than 100 colonies/dish for *yeast* detection.

**iii. Spread Plate Method**

For the spread coating method, take 0,05-0,2 mL of the test sample suspension on the surface of the solid agar medium, then spread it out using spreader. For bacterial detection, use TSA medium and for *yeast* detection use SDA medium that has previously been added with antibiotics. Then incubate for approximately 5 days at a temperature of 30-35°C for bacterial detection and at a temperature of 20-25°C.

**D. Factors affecting the bioburden test**

According to (Filaire et al., 2023), there are several factors that can affect the results of bioburden testing on a medical product:

1. Colony forming units (CFU) are not actual cell counts (individual cells are rarely found in nature, which causes CFU to be an underestimate of the number of microorganism present).
2. Incubation conditions such as temperature as well as time and the physical condition of the organism (stressed or sublethally damaged due to high temperature, humidity, ionic strength, pH extremes, osmotic shocks related to liquids, and antimicrobial residues).
3. Nutritional requirements of microorganism and sample dilution errors.

**Endotoxin Test**

**A. General Description**

Endotoxins are the most common pyrogens found in medical devices and can cause significant risks to patients if undetected. Pyrogens are substances that can trigger fever and can be dangerous or even fatal if given to humans above certain concentrations (Schlottrerer & Wolff, 1996). High levels of endotoxins can enter the bloodstream through medical devices, causing adverse reactions such as hemorrhagic shock, diarrhea, meningitis, fever, altered resistance to bacterial infections, rapid drops in blood pressure, and a variety of other serious biological effects (Foster & Kellum, 2023). Endotoxins are usually part of the membrane-bound lipopolysaccharides in the cell walls of Gram-negative bacteria. The toxicity of these lipopolysaccharides is due to the Lipid A component of the lipopolysaccharides (Foster & Kellum, 2023).

Bacteria produce Lipid A from a disaccharide molecule, myristic acid, and unique fatty acids. The substance remains highly toxic because it fails to trigger an adaptive immune response which leaves patients vulnerable to repeated endotoxin-mediated shock incidents. The

core oligosaccharide serves as a genus-specific characteristic consisting of oligosaccharide units together with phosphate and amino acids.The interaction between lipopolysaccharides and LPS-binding protein with CD14 and TLR-4 on CD4 cells results in NF-κB activation (Mohsen, 2023). The transcription of cytokine genes which encompass TNF-α and IL-1 is controlled by NF-κB. Systemic LPS levels in high concentrations initiate dysregulated cytokine release and complement activation which together can cause septic shock and multi-organ failure (Sheehan et al., 2022).

The study explored endotoxin reduction using standard sterilization methods including ionizing radiation and both dry and moist heat as well as ethylene oxide sterilization. The reduction of endotoxin activity after sterilization treatments depended on both the endotoxin source and the substrate material A variety of materials and types show significant reductions in endotoxin activity when exposed to moist heat through steam. Many materials show effective endotoxin activity reduction when exposed to dry heat. The depyrogenation example represents standard practice even though the conditions tested were below normal depyrogenation cycle parameters. Research findings indicate that endotoxin becomes inactive once temperatures exceed 150°C. Endogenous Oxygen treatment reduces endotoxin concentrations yet the substrate material has a stronger influence on its performance than other methods. E-beam sterilization reduces endotoxin activity highly on metallic surfaces while showing reduced effectiveness on nonmetallic materials (Kimble et al., 2023).

Bacterial Endotoxin Test (BET) is a test to detect or measure endotoxins from Gram-negative bacteria (Baker et al., 2023). Endotoxin test, also known as LAL (Limulus Amebocyte Lysate), is an essential to pharmaceutical microbiology testing. The LAL test debuted in the early 1970s. Initially, it offered a more sensitive, semi-quantitative method of assessing concentrations of natural ambient endotoxins (Dubczak et al., 2021). Any medical device that comes into direct or indirect contact with the intravascular, intralymphatic, intrathecal, and/or intraocular systems must undergo bacterial endotoxin testing to ensure the device's endotoxin content is below the medical device and pharmaceutical manufacturing device specifications (Tamura et al., 2021). If these endotoxin levels are present in medical devices or pharmaceuticals, they can cause serious harm or even death to patients depending on the type of patient contact. Devices that come into contact with the circulatory system, cerebrospinal fluid, eyes, implants, and other body parts that may be susceptible to endotoxins must be monitored regularly to make sure the patient is safe (Marius et al., 2020).

## B. Standar uji endotoksin untuk perangkat medis

Details regarding endotoxin testing and limits for medical devices can be found in the Pharmacopoeia (USP<85>, USP<161>, USP<1085>, EP 2.6.14, etc.). Furthermore, (Marius et al., 2020). noted that endotoxin limits for a device are determined by the type of device used as follows:

**Table 2. Of Endotoxin Limits In Medical Devices (Marius et al., 2020)**

| Device Type | Limit (Endotoxin Units/Device) |
|---|---|
| **Circulation Contact** | < 20 |
| **Cerebrospinal Fluid Contact** | < 2,15 |
| **Intraocular Device** | < 0,2 |

Endotoxins usually remain even though the bacteria-producing endotoxins have died. In living bacteria, endotoxins can continue to be released by cells. Given the very small size of endotoxins, filtration methods are generally ineffective for removing endotoxins. Strong acids, strong bases, or high temperatures may be needed to reduce endotoxin contamination in a product, but these methods can have adverse effects on certain medical device component materials (Marius et al., 2020).

## C. Endotoksin Testing Method

In carrying out the endotoxin testing method, several reagents will be used. The reagent commonly used is Limulus Amebocyte Lysate (LAL). LAL is an extract from horseshoe crab blood cells which are very sensitive to endotoxins (Marius et al., 2020). The following are some reagents that are often used in endotoxin testing.

1. *Limulus Amebocyte Lysate* (LAL): Used in gel-clot, turbidimetric, and chromogenic methods to detect endotoxins.
2. *Control Standard Endotoxin* (CSE): Used as a control standard to ensure test accuracy.
3. Turbidimetri Reagent: Used in turbidimetric methods to measure the turbidity of samples containing endotoxin.
4. Chromogenic Reagent: Used in chromogenic methods to detect color changes that occur in the presence of endotoxin.

The above reagents are used in endotoxin testing to ensure that pharmaceutical and medical products are free from harmful endotoxin contamination. Bacterial Endotoxin testing utilizes a combination of a liquid or liquid extract from a product/device combined with a Limulus Amebocyte Lysate (LAL) reagent to detect or measure bacterial endotoxin in samples through several methods.

Extraction for endotoxin testing is usually performed on the part of the device that comes into contact with the patient (direct or indirect contact) or that is labeled non-pyrogenic. Extraction is usually performed for 15 minutes at 37°C or one hour with 37°C water extracted at room temperature. Intraocular products may require extraction for one hour at 37°C with agitation. Endotoxin testing can be performed before or after the sterilization process. The risk of endotoxin testing before sterilization comes from the potential for changes in the Gram-negative bioburden between the time of testing and the time of sterilization which can affect endotoxin levels. There is a high risk of increased endotoxin levels in samples that contain water or nutrients that are needed for bacteria or fungi to grow (Spoladore et al., 2021).

Various analytical methods can be performed based on modifications to the Limulus Amebocyte Lysate (LAL) reagent. The analytical method chosen for endotoxin testing must be validated to ensure that there is no inhibition or increase in endotoxin levels in the test performed. Therefore, for all methodologies that will be used later, it is necessary to perform a positive product control (PPC) sample test to measure the potential for inhibition or increase in the sample extraction solution (Spoladore et al., 2021). The following is a comparison of endotoxin testing methods that can be performed in testing medical devices:

### a. Gel-Clot Method

The Gel-Clot method is one of the endotoxin testing methods that uses Limulus Amebocyte Lysate (LAL) to detect the presence of endotoxin in a sample. The working principle of this method is that LAL (Limulus Amebocyte Lysate) will form a clot (gel) when exposed to endotoxin. The LAL (Limulus Amebocyte Lysate) reagent itself comes from the blood of ancient animals, namely hermit crabs (Limulus polyphemus) which still exist today, this animal is a type of marine arthropod commonly known as horseshoe crab. The blood of

this animal contains blood cells called amoebocytes, which are very sensitive to bacterial endotoxins, such as those found in the cell walls of gram-negative bacteria. When endotoxin is present, these amebocyte cells will react, producing a color change or clotting, which is then used to detect the presence of endotoxin. Because of the amoebocyte content in their blood, hermit crabs can adapt and survive until now (Hashmi & Thakur, 2019).

In endotoxin test, the sample volume used usually ranges from 50-100 µL, depending on the product specifications and methods used. This method is generally used for parenteral pharmaceutical products, injectable preparations, and other biological materials that must be free from endotoxin contamination. Samples are taken after the sterilization process and before the product is put into the final packaging. This is important to ensure that the sample being tested truly reflects the condition of the product to be used. In this method, the sample is filled into a well-plate or microplate that is non-pyrogenic. Then LAL (Limulus Amebocyte Lysate) is added to each well containing the sample. LAL (Limulus Amebocyte Lysate) with the bound sample will show a clot when the sample contains endotoxin. In this method, there needs to be positive and negative controls in the test, positive control is carried out to ensure that the reagent used, namely LAL (Limulus Amebocyte Lysate) can react and cause clotting when bound to CSE (Control Standard Endotoxin), while the negative control well contains only the LAL (Limulus Amebocyte Lysate) reagent. Sample incubation after testing is incubated at 37 ° C for 60 minutes in a water bath (Hashmi & Thakur, 2019).

After incubation for 60 minutes, the results are analyzed. Where the analysis in this method is qualitative by observing the formation of gel in each well by turning the well. If a gel is formed, the result is positive, meaning there is endotoxin in the sample. If no gel is formed, the solution in the well plate or microplate will dissolve when the well is turned over and show a negative result for endotoxin in the sample.

The advantage of the Gel-Clot method is that the testing process is relatively simple and the results are obtained quickly. In addition, this method does not require sophisticated equipment or high costs. However, besides the advantages, this method also has disadvantages, namely it has a lower level of sensitivity compared to other methods such as chromogenic or turbidimetry, and the results obtained are not quantitative or only provide positive or negative results without providing accurate values (Hashmi & Thakur, 2019).

**b. Turbidimetri Method**

The Kinetic Turbidimetry method is a testing technique that measures changes in turbidity in solutions containing endotoxins. The working principle of this method is that endotoxins will cause the formation of a gel that produces changes in turbidity that can be measured kinetically. The working principle of this method is almost the same as the Gel-Clot test method, except that there is an additional test on the sample incubation results. In this method, turbidity is measured using a spectrophotometer or turbidimeter to measure changes in turbidity during incubation (Xu et al., 2024).

The samples used in this method are the same as the Gel-Clot test, usually ranging from 50-100 µL, depending on the specifications of the product to be tested. The types of products used in this method are parenteral pharmaceutical products, injection preparations, and other biological materials that must be free from endotoxin contamination. However, endotoxin testing for all products that require this test can be carried out either by the Gel-Clot test method or other tests, depending on the data required. If the results required are only qualitative, then the Gel-Clot method or the Turbidimetry method is sufficient to use.

Analysis in this method can be quantitative or qualitative by observing the turbidity of the sample. Changes in turbidity that occur during incubation are interpreted to determine the

presence of endotoxin in the sample. The Kinetic Turbidimetry method only produces positive or negative data based on changes in turbidity. The advantage of the Kinetic Turbidimetry method is that it has a slightly higher sensitivity value than the Gel-Clot method because it can detect low levels of endotoxin (Xu et al., 2024). Although by observing the presence or absence of turbidity in the post-incubation sample, this method can be measured quantitatively by calculating the absorbance value or measuring the level of turbidity in the sample to give the detail information about the levels of endotoxin in the sample. Meanwhile, the disadvantage of the Kinetic Turbidimetry method is that it has a more complicated and more complex testing process than the Gel-Clot method because it requires more sophisticated equipment to measure the desired level of turbidity (Hashmi & Thakur, 2019).

**c. Kinetic Chromogenic Method**

The Kinetic Chromogenic Method is an endotoxin testing technique that measures the color change (chromogenic) produced by the reaction between the endotoxin and the Limulus Amebocyte Lysate (LAL) reagent. This reaction produces a colored product whose intensity can be measured kinetically. The purpose of this method is to identify and quantify the level of endotoxin in a sample and provide accurate quantitative results regarding the concentration of endotoxin (Barro et al., 2021).

The sample volume used is usually between 50-100 µL, depending on the product specifications and the method used. This kinetic chromogenic method is used to test various types of products such as:

- Pharmaceutical products: Injectable preparations, infusions, and other parenteral products.
- Biological materials: Vaccines, recombinant proteins, and other biological products.
- Medical devices: Equipment that comes into direct contact with blood or body tissue.

Liquid samples usually have a shorter shelf life and need to be tested within 24 hours. Solid or Semi-Solid samples have a slightly longer shelf life but should still be tested within 24-48 hours. In the test, samples are taken after the sterilization process and before the product is put into the final packaging. This method uses the same reagents as other methods, namely using the Limulus Amebocyte Lysate (LAL) reagent, which is the main reagent because it has a level of sensitivity to the presence of endotoxins Because of the amoebocyte content in their blood, hermit crabs can adapt and survive until now (Marius et al., 2020). However, in addition to these reagents in general, this method requires an additional reagent, namely a chromogenic reagent, which will later react with endotoxin to produce a color change (Barro et al., 2021).

In addition, this method also requires a microtiter reader which is used to measure the intensity of the color that will be produced. In this method, after the incubation process at 37 ° C for a specified time (usually 60 minutes). Furthermore, kinetic measurements are carried out using a microtiter reader to measure changes in color intensity periodically during incubation.

Data analysis carried out in this method is by observing color changes to determine the concentration of endotoxin in the sample. The test results are interpreted based on changes in color intensity measured by a spectrophotometer. This data is then used to calculate the concentration of endotoxin using a predetermined standard curve. The results are measured as changes in absorbance (ΔA) which is directly related to the endotoxin concentration (Endotoxin Unit or EU). An endotoxin standard curve is created to convert ΔA to EU/mL values. The advantage of the Kinetic Chromogenic method is that it has a high level of sensitivity. This method is very sensitive and can detect low concentrations of endotoxin and produce data that can be measured quantitatively. However, in the process, this method is more complicated because it needs more sophisticated equipment and more complex procedures than other

methods. In addition, this method requires higher costs because it requires special reagents and analysis devices (Barro et al., 2021).

## Conclusion

Various testing methods for detecting microbial contamination in medical devices are conducted to minimize risks and prevent failures in the production process. Sterilization testing is a crucial step in ensuring that a product is free from pathogenic microorganisms. Commonly used methods include autoclaving, radiation, and chemical sterilization. Meanwhile, bioburden refers to the total number of microorganisms present on a product before sterilization. Understanding bioburden levels is essential, as a high bioburden may indicate potential contamination risks and help assess the effectiveness of the sterilization process.

Endotoxins are byproducts of Gram-negative bacterial cell walls that can trigger harmful reactions in humans. Endotoxin testing, such as the Limulus Amebocyte Lysate (LAL) assay, is essential for products that come into contact with the human body. Controlling and testing endotoxin levels help ensure the safety of medical products, particularly in healthcare settings.

In conclusion, providing a comprehensive understanding of microbiological testing of medical devices—such as sterility, bioburden, and endotoxin assessments—plays a crucial role in supporting the sustainability of the healthcare and pharmaceutical industries. Equally important is the introduction of these microbiological testing concepts to students in vocational health schools (SMK), as it equips them with essential knowledge and practical skills to help ensure the safety of medical devices in the future. Through this review, graduates of vocational health programs are expected to contribute meaningfully to maintaining patient safety standards and strengthening the overall credibility of the healthcare industry in the long term.

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