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# Detection Limit and Sensitivity of *Staphylococcus aureus* Detection in Pharmaceutical Products

# (Batas Deteksi dan Sensitivitas Metode Deteksi *Staphylococcus aureus* dalam Sediaan Obat)

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**Abstract:** *Stapylococcus aureus* is a pathogen that should be absent in pharmaceutical products. Contamination of certain microorganisms can potentially reduce or inactivate therapeutic activity, affect the stability, efficacy and cause infection of the patient. Microbial detection methods must be valid and sensitive to detect the contamination of microorganisms at low concentrations. The standard method of *S. aureus* refers to the Indonesian Pharmacopoeia (FI). Limit of Detection (LOD) and sensitivity of the method in general is not stated in FI. This study aims to determine LOD and sensitivity of *S. aureus* method on pharmaceutical products with route of administration in cutan, oromucosal, gingival, auricular, vaginal and oral based on Indonesian Pharmacopoeia. Experiments used nine pharmaceutical products representing six dosage forms contaminated with *S. aureus* ATCC 6538 with three level concentrations of ±1, ±3, and ±5 CFU per g atau mL sample. LOD and sensitivity were determined and analyzed descriptively. This study shows that the detection limit is 1-3 colonies per g or mL sample and a sensitivity of 100%. Results of this study can be used as reference for LOD value in the validation or verification process of the *S. aureus* detection method in the laboratory to ensure validity of the methods used.

**Keywords:** Limit of detection, pharmaceutical product, sensitivity, *Staphylococcus aureus*

**Abstrak:** *Staphylococcus aureus* merupakan bakteri patogen yang dipersyaratkan tidak ada dalam sediaan farmasi. Kontaminasi mikroba dalam sediaan farmasi dapat menginaktifkan aktivitas terapeutik, mempengaruhi stabilitas, efikasi obat, dan menyebabkan infeksi pasien. Metode standar untuk deteksi *S.aureus* pada produk farmasi mengacu pada Farmakope Indonesia (FI). FI tidak menyatakan batas deteksi dan sensitivitas metode secara umum, sehingga nilai rentang *Limit of Detection* (LOD) dan sensitivitas metode perlu ditetapkan dalam berbagai matriks yang diuji. Penelitian ini bertujuan untuk mengetahui nilai rentang LOD dan sensitivitas metode pengujian *S. aureus* pada produk obat pengunaan pada kulit, oromukosa, gingival, *auricular,* vaginal dan oral sesuai FI. Deteksi *S. aureus* telah dilakukan terhadap sembilan produk obat yang mewakili enam matrik sediaan yang dicemari dengan *S. aureus* ATCC 6538 pada tiga tingkat konsentrasi ±1, ±3, dan ±5 koloni per g atau mL sampel. nilai rentang LOD dan sensitivitas metode ditetapkan dan dianalisis secara deskriptif kualitatif. Metode deteksi *S. aureus* sesuai farmakope dapat mendeteksi *S. aureus* dalam 9 sediaan obat dengan batas deteksi antara 1-3 koloni per g atau mL sampel dengan sensitivitas 100%. Hasil penelitian ini dapat digunakan sebagai acuan nilai LOD pada proses validasi/verifikasi metode deteksi *S. aureus* dalam produk obat oleh laboratorium untuk menjamin keabsahan metode yang digunakan.

**Kata kunci:** *Limit of detection*, sediaan obat, sensitivitas, *Staphylococcus aureus*

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## INTRODUCTION

PHARMACEUTICAL products consist of two groups, namely sterile and non-sterile products. Both sterile and non-sterile products available in the markets in Indonesia must meet the microbiological quality requirements based on Indonesian Pharmacopoeia Monograph VI (FI VI). Microbiological quality requirements for non-sterile drug products include Total aerobic microbial count (TAMC), Total Yeast and Mold count (TYMC), bile tolerant Gram negative bacteria*, Escherichia coli, Pseudomonas aeruginosa*, *Salmonella* spp.*, Clostridium* spp., *Candida albicans,* dan *Staphylococcus aureus*[1]. Contamination of certain microorganisms may change the physico-chemical properties of drugs, potentially reduce and inactivate therapeutic activity and to be more toxic compounds[2] and dangerous to patient health[3].

Non-sterile pharmaceutical products can be classified based on the route of administration use such as cutaneous, oral, oromucosal, gingival, vaginal, and auricular preparations which have the same microbiological quality criteria as negative *S. aureus* per gram or mL sample[4].

*Staphylococcus aureus* is a type of pathogenic bacteria in humans. This bacterium is Gram positive, coccus. *Staphylococcus aureus* causes infections such as respiratory infections, dermatitis, and mastitis. These bacteria can also be a major cause of endocarditis, bacteremia, osteomyelitis, and soft tissue infections. There is a variant of *S. aureus* that is resistant to antibiotics and is known as Methicilin-Resistant *Staphylococcus aureus* (MRSA). MRSA is still a threat in the world of health, with high morbidity and mortality rates. The successful cure of MRSA infection remains a challenge, and requires evaluation of the effectiveness of new antimicrobials[5,6]. This antibiotic-resistant bacteria has been reported to contaminate ultrasound gel in healthcare facilities[7]. Based on reports, 80% of *S. aureus* can survive on ultrasound gel for 1 hour, has the potential for cross-contamination between patients and allows patient infection in hospitals (health care-associated infection)[8,9].

In pharmaceutical preparations*, S. aureus* is one of the indicator bacteria whose presence is considered as a indicator of product contamination. *S. aureus* contamination is often found in sterile and non-sterile products resulting in product rejection[6,10]. *S. aureus* is required to be negative per gram or mL of sample, so a method is needed that can detect bacteria at low concentrations. The Indonesian Pharmacopoeia VI (2020) states that a microbial detection method, including *S. aureus*, must be able to detect microbes not more than 100 colonies, but the actual limit of detection has never been determined quantitatively and many variables can affect microbial recovery in preparations. These variables include the characteristics of the preparations tested, the physical conditions of the microorganisms, the nutritional needs of the microorganisms, the media and the incubation condition[4].

According to the International Conference on Harmonisation[11], Limit of Detection (LOD) is the smallest amount of analyte in a sample that can be detected but is not important to measure under experimental conditions. In qualitative microbiology, the LOD of a microorganism detection testing method is defined as the smallest number of microorganisms in a certain volume of sample that can be detected under certain experimental conditions[4].

The culture method is the standard method for detecting *S. aureus* in the products. Several studies have reported the modification of standard method with alternative Polymerase Chain Reaction (PCR) method to increase the sensitivity of *S. aureus* detection in food products[12,13]. Likewise, MRSA from clinical samples can be detected using both media culture and molecular methods using PCR[14,15]. In pharmaceutical products, the use of PCR as a detection method for *S. aureus* has been reported at 100 colonies/mL sample[16]. The results of a subsequent study[17] showed that contaminated raw materials and pharmaceutical preparations with a bacterial concentration of less than 2 colonies/g could not be detected by conventional culture methods, but could be detected molecularly using multiplex PCR. The culture method is the gold standard for detecting indicator microoragnisms in raw materials and pharmaceutical preparations. It is necessary to determine the range of LOD values and the sensitivity of the culture method in the various matrices tested. Previous research[18] has reported the range of LOD and the sensitivity of the *P. aeruginosa* test method on various pharmaceuticals preparations. This study aims to determine the range of LOD and the sensitivity of the *S. aureus* detection method in 9 pharmaceutical products with of with different dosage forms.The results of this study can be used as a reference for the LOD value in the process of validation or verifcation the *S. aureus* detection method in pharmaceuticals preparations in each test laboratory.

## MATERIALS AND METHODS

**MATERIALS.** The materials used in the research are potassium dihydrogen phosphate (Merck, Darmstadt, Germany) and sodium hydroxide (Merck, Darmstadt, Germany) to prepare a phosphate buffer solution (PBS) pH 7.2, polysorbate 80 (Merck, Darmstadt, Germany), lecithin (Merck, Darmstadt, Germany),

Microbial growth Media (Soybean Casein Digest Broth (SCDB) (Merck, Darmstadt, Germany), Mannitol Salt Agar (MSA) (Merck, Darmstadt, Germany), Soybean-Casein Digest Agar (SCDA) (Merck, Darmstadt, Germany)), GP card VITEK® (BioMerieux, Durham, North Carolina, United States), Pharmaceuticals products include cutaneous use (acyclovir, hydrocortisone, ketoconazole dan fluocinolone acetonide creams dosage forms), oromucosal use (triamcinolone acetonide ointment), gingival use (mouthwash liquids contain benzidamine hydrocloride), *auricular use* (sodium docusate drops), vaginal insert tablet (nystatin) dan oral use (alumina and magnesia suspension), and microbial standard *S. aureus* ATCC 6538 (Microbiologics, St. Cloud, Minnesota, United States).

**Tools.** Instrument used in this research is VITEK® 2 *Compact System* (BioMerieux, Durham, North Carolina United States).

**METHODS. Sample Preparation.** Samples were prepared acording to the 6th Indonesian Pharmacopoeia Edition[1]. Pharmaceuticals preparations with ointments, creams, and tablets dosage forms were aseptically weighed as 10 g, while for the suspension and liquid dosage forms were pipetted as 10 mL, put into a suitable sterile container, then added phosphate buffer solution pH 7.2 to obtain a $10^{-1}$ dilution. For fatty preparations (creams and ointments), the sample was dissolved with sterile polysorbate 80 surfactant (1 mL in 10 g sample), added PBS pH 7.2, to obtain a suspension with a dilution of $10^{-1}$ and shaken until homogeneous. Samples containing preservatives, neutralized with polysorbate 80 and lecithin mixed into PBS with a concentration of polysorbate 80 (30 g/L) and lecithin (3 g/L)

**Preparation of Spiked Samples.** The preparation of the bacterial suspension for spikes was based on calculating number of *S. aureus* ATCC 6538 by TAMC method using McFarland standard 1 and a UV-Vis Spectrophotometer λ 580 nm (42% Transmittance). One loop of *S. aureus* culture were inoculated onto the surface of the SCDA agar plate and incubated at 37°C for 24 hours, then *S. aureus* 1 McFarland suspension was made, the turbidity of the suspension was measured at ±42% Transmittance and the number of colonies was counted using the TAMC method. To obtain the concentrations of ±1, ±3, and ±5 colonies per g or mL of sample, *S. aureus* inoculums containing 10, 30 and 50 colonies were added to 10 g or 10 mL of the sample. To obtain the number of colonies, a serial dilution was carried out from *S. aureus* suspension stock (±42%T) using PBS pH 7.2.

**LOD Determination.** LOD determination was carried out at 3 concentration levels of *S. aureus* ±1, ±3, and ±5 colonies per g or mL sample. Each concentration level was carried out as 6 test replications for each sample. The sample suspension that had been contaminated with *S. aureus* suspension at the spiked sample preparation step was pipetted as 10 mL and inoculated into 90 mL of SCDB enrichment medium and then incubated for 24 hours. After incubation, a loop of suspension was inoculated into the MSA plate agar medium using 3 mm diameter loops (equivalent to 10 µL). MSA medium was incubated at 32.5 °C for 24 hours. Suspected growth of yellow colonies of *S. aureus* was identified using the VITEK® 2 Compact System instrument (Durham, North Carolina United States). The LOD value was determined based on the smallest *S. aureus* concentration in each sample that could still be detected in all replicates[4].

**Sensitivity Determination.** Sensitivity was determined from 6 replications of the *S. aureus* detection test on each pharmaceuticals preparation contaminated with *S. aureus* at the LOD value. Sensitivity was determined from the number of bacterial cultures isolated from spiked samples that were confirmed positive for *S. aureus*. Calculation of the sensitivity value is calculated using the following formula[19]:

$$\text{Sensitivity (\%)} = \frac{\textit{Number of True Positive} \text{ (TP)}}{(\textit{Number of True Positive} \text{ (TP)} + (\textit{False Negative} \text{ (FN)})} \times 100\%$$

True positives are the number of test replications that are confirmed positive from positive suspects, while false negatives are the number of test replicates that are confirmed positive from negative suspects.

**Data Analysis.** Data analysis was performed with descriptive statistical analysis. The analytical descriptive method is a method that describe or give an overview of the object under study through data or samples that have been collected as they are without conducting analysis and making general conclusions[20].

## RESULTS AND DISCUSSION

**Number of *S. aureus* ATCC 6538 Count.** To obtain spike concentrations, the suspension of *S. aureus* bacteria stock are calculated using TAMC method. The results of the TAMC test for *S. aureus* (42% Transmittance, λ 580 nm are as follows (Table 1).

Table 1 described that the growth of *S. aureus* are proportional from low to high dilution. At low dilution levels, the growth of *S. aureus* colonies on Petri dishes was too much to count (TMTC). Colonies can be counted starting from the $10^{-6}$ dilution until a unit value is obtained at $10^{-8,}$ while at the $10^{-9}$ dilution no colony growth to be seen.

**Table 1. Data number of *Staphylococcus aureus* (42% Transmitan, λ 580 nm).**

| Incubation time (hour) | Number of colonies in each dilution | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|
| | $10^{-1}$ | $10^{-2}$ | $10^{-3}$ | $10^{-4}$ | $10^{-5}$ | $10^{-6}$ | $10^{-7}$ | $10^{-8}$ | $10^{-9}$ |
| | ~ | ~ | ~ | ~ | ~ | 164 | 16 | 1 | 0 |
| 24 | ~ | ~ | ~ | ~ | ~ | 170 | 18 | 0 | 0 |
| | ~ | ~ | ~ | ~ | ~ | 203 | 17 | 1 | 0 |
| 48 | ~ | ~ | ~ | ~ | ~ | 209 | 23 | 0 | 0 |

Note: ~ means "too many to count" (TMTC)

The TAMC value is the average of the number of colonies that grow at the dilution which shows the highest colony number not more than 250 colonies. In this study, the TAMC value of *S. aureus* was calculated from the average of colonies that grew at $10^{-6}$ dilution, and the TAMC value was $2.1x10^8$ colonies/mL.

This TAMC value is then used as a reference value for contaminating each pharmaceuticals sample with concentrations of ±1, ±3, and ±5 colonies per g or mL of sample to determine the LOD value for each preparation.

**LOD Determination.** Detection of *S. aureus* has been carried out on 9 pharmaceutical preparations with the active ingredients acyclovir, hydrocortisone, ketoconazole, fluocinolone acetonide, triamcinolone acetonide, alumina and magnesia, benzidamine hydrochloride, sodium docusate and nystatin which were spiked with *S. aureus* with concentrations close to one colony, namely ±1, ±3, and ±5 colonies/g or mL of sample. The testing were performed in 6 replication for each concentration in each samples. Suspected *S. aureus* colonies were yellow colonies on MSA plate media. Suspected *S. aureus* colonies were yellow colonies on MSA plate agar media (Figure 1).

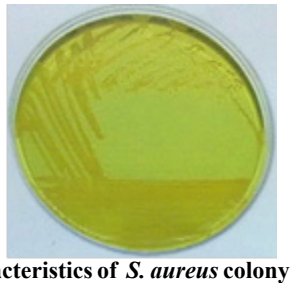

**Figure 1. Characteristics of *S. aureus* colony on MSA media.**

The growth of isolated bacteria from each sample was biochemically confirmed using the VITEK® 2 Compact System (Biomeriux, Durham, North Carolina United States) instrument and showed the same results, excellent identification (99%). The number of replicates showing the isolate confirmed as *S. aureus* in each sample was calculated to determine the LOD value (Table 2). This study showed a range of LOD are 1 to 3 colonies per g or mL of pharmaceutical sample (Table 3).

The four cutaneous dosage form showed the same LOD value, 1 colony/g. These preparations were acyclovir cream, hydrocortisone cream, ketoconazole cream and fluocinolone acetonide cream respectively. In the preparation steps, polysorbate 80 and lecithin were added as neutralizers for preservatives commonly used in skin preparations[21]. With the addition of polysorbate 80 (30 g/L) and lecithin (3 g/L), the cutaneouse dosage form had no longer antimicrobial activity, so that *S. aureus* with a concentration of 1 colony/g was able to grow in all replicates (Table 2). The LOD value obtained in this sample is lower than previous studies with neutralizers of 4% polysorbate 20 and 0.5% lecithin, which had LOD greater than 2 colonies/g samples of raw materials and medicinal preparations[17].

Oromucosal preparations are drugs that are given through the mucosa in the oral cavity, while oral preparations are drugs that are administered through the mouth and enter the body through the digestive tract[22]. The matrix of the oromucosa preparation used in this study was in the form of an ointment, polysorbate 80 was added as a neutralizer and emulsifier during preparation step of the oromucosa preparation. Table 2 describes *S. aureus* with a concentration of 1 colony/g of oromucosa preparation samples could grow and was confirmed as *S. aureus* in all replicates, consequently the LOD value in this oromucosa preparation was 1 colony/g. Table 2 also shows that at a concentration of 1 colony/mL of oral sample, the recovery of *S. aureus* was only 50% (3 of 6 replicates), while the growth of this bacterium at a concentration of 3 colonies/mL could be seen in all replicates hence the LOD value for the preparation was determined to oral suspension matrix at a value of 3 colonies/mL sample. The oral preparation samples used are in the form of a suspension containing alumina and magnesia which are soluble in water so that they do not require special preparation when preparing the samples.

Auricular preparations with matrix of ear drops containing docusate sodium showed an LOD value of 3 colonies/mL. The ear drops in this study contained 5 mg docusate sodium. At the preparation step of ear drops was optimized by adding polysorbate 80 as a neutralizer, but there was no difference in the growth of *S. aureus* in this preparation so it had no effect on

**Table 2. Number of replications confirmed as positif *S. aureus* on spike concentration of ±1, ±3, ±5 colony per g or mL sample.**

| Spiked Samples | | | Number of replicates confirmed positive *S. aureus* at spike concentration per g or mL of sample | | |
|---|---|---|---|---|---|
| Route of Administration | Dosage forms | Active ingredients | ±1 colony | ±3 colony | ±5 colony |
| Cutaneous use | Cream | acyclovir | 6 | 6 | 6 |
| Cutaneous use | Cream | hidrocortisone | 6 | 6 | 6 |
| Cutaneous use | Cream | ketoconazole | 6 | 6 | 6 |
| Cutaneous use | Cream | fluocinolone acetonide | 6 | 6 | 6 |
| Oromucosal use | Ointment | triamcinolone acetonide | 6 | 6 | 6 |
| Oral use | Suspension | alumina and magnesia | 3 | 6 | 6 |
| Gingival use | Liquid | benzidamine hidrocloride | 1 | 6 | 6 |
| Auricular use | Drops | sodium docusate | 0 | 6 | 6 |
| Vaginal use | Insert Tablet | nystatine | 4 | 6 | 6 |

**Table 3. Detection limit of S. *aureus* testing methods in pharmaceutical products.**

| Route of Administration | Dosage forms | Active ingredients | LOD (n=6) |
|---|---|---|---|
| Cutaneous use | Cream | acyclovir | 1 CFU/g |
| Cutaneous use | Cream | hidrocortisone | 1 CFU/g |
| Cutaneous use | Cream | ketoconazole | 1 CFU/g |
| Cutaneous use | Cream | fluocinolone acetonide | 1 CFU /g |
| Oromucosal use | Ointment | triamcinolone acetonide | 1 CFU /g |
| Oral use | Suspension | alumina and magnesia | 3 CFU/mL |
| Gingival use | Liquid | benzidamine hidrocloride | 3 CFU/g |
| Auricular use | Drops | sodium docusate | 3 CFU/g |
| Vaginal use | Insert Tablet | nystatine | 3 CFU/mL |

the LOD value. Sodium docusate with a concentration of 3 mg/mL of the active substance in the drug can inhibit the growth of *S. aureus*[23].

For vaginal preparations containing nystatin, as shown in Table 1, samples contaminated with a concentration of *S. aureus* 3 colonies/g showed bacterial growth and were confirmed positive in all replicates, whereas at lower concentrations, *S. aureus* was only able to grow as many as 4 replications (66.7%). The LOD value was determined at a concentration of 3 colonies/g. This value is still included in the lower concentration of the three concentration levels determined in the study. Nystatin is an effective antibiotic for molds and yeasts but not for bacteria, viruses, and protozoa[24].

Gingival preparations are preparations that are applied to the gingiva or gums. The gingival preparation used in this study was in the form of a liquid mouthwash containing benzidamine hydrochloride. Samples contaminated with *S. aureus* with a concentration of 1 colony/mL sample only showed growth in one replicate. At a concentration of 3 colonies/mL, the growth of *S. aureus* was seen in all replicates. Benzidamine hydrochloride is a non-steroidal anti-inflammatory drug (NSAID) with analgesic properties and exhibits antimicrobial properties[25]. The antimicrobial properties of this active substance may inhibited the growth of *S. aureus* at a concentration of ±1 colony/mL, so that the LOD value was obtained at 3 colonies/mL. LOD of

3 colonies/mL still meets the suitability requirements of the microbiological test method.

Some of the drug dosage forms used in this study contained preservatives/antimicrobial substances. A specific treatment was required to neutralize the antimicrobial substances. Techniques for neutralizing antimicrobial substances in pharmaceutical preparations can be carried out through the process of diluting samples, using chemical substances, and a combination of these two techniques. Neutralizing agents are used to neutralize the activity of antimicrobial compounds including Sodium bisulfite, lecithin, polysorbate, thioglycolate, thiosulfate, Mg or Ca ions or by dilution. The use of neutralizing agents must be effective in eliminating antimicrobial activity and not be toxic[1,3]. The neutralizing agents used in this study were polysorbate 80, lecithin, a combination of polysorbate 80 and lecithin and dilution.

Oromucosal preparations with an ointment form were prepared using the neutralizing agent polysorbate 80, while cream dosage form used polysorbate 80 and lecithin because there is a possibility that these preparations contain inhibitory substances parabens or quaternary ammonium compounds which can be neutralized with polysorbate 80 and lecithin. In this study the combination of polysorbate 80 (30 g/L) and lecithin (3 g/L) was effective for recovering contaminated *S. aureus* in cutaneous and oromucosal preparations. The use of this type of chemical neutralizer was also reported to be effective for the recovery of various bacteria and fungi with low concentrations in the sample[26]. The ear drop preparations are neutralized by dilution because the sodium docusate content in the preparation is not effectively neutralized by adding a neutralizing agent.

The existence of a neutralization process of antimicrobial activity in drug samples can increase the detection limit of microbiological test methods. The LOD range of the *S. aureus* detection method for drug products obtained in this study had values between 1-3 colonies per g or mL sample. This result corresponds to the LOD value specified for the microbiological test according to the test method validation and verification guidelines[27].

**Sensivity Determination.** The sensitivity calculation was carried out using data from 6 test replicates for each drug preparation contaminated with *S. aureus* at the LOD value. For cutaneous and oromucosal preparations, at an LOD value of 1 colony/g sample, *S. aureus* growth was seen in all replicates (Table 2), consequently the sensitivity value was 100%. Likewise with oral, gingival, auricular and vaginal preparations, *S. aureus* growth was found in all replicates with *S. aureus* spiked concentrations at the detection limit of 3 colonies/g or mL sample. Sensitivity indicates the ability of the method to detect analytes, in this case is *S. aureus*[19]. This study shows that the *S. aureus* detection method on several drug dosage forms with different matrices shows the same sensitivity as 100% (Table 4).

A sensitivity of 100% indicated that the *S. aureus detection* method used in this study have capability to detect *S. aureus* in various drug preparation matrices. *S. aureus* was able to grow and was confirmed positive in all replicates of each spiked sample with a concentration at its detection limit. This also shows that there were no false negatives found in the detection method used in the study[19]. The *S. aureus* detection method used in this study is sensitive and valid for all matrices of the drug preparation.

## CONCLUSION

The LOD of the *S. aureus* detection method in nine pharmaceuticals preparations can vary depending on the differences in the dosage forms and active substance of each drug preparation, with values ranging from 1-3 colonies per g or mL sample. The *S. aureus*

**Table 4. Sensitivity of *S. aureus* testing methods in pharmaceutical products.**

| Route of Administration | Dosage forms | Active ingredients | Sensitivity (n=6) |
|---|---|---|---|
| Cutaneous use | Cream | acyclovir | 100% |
| Cutaneous use | Cream | hidrocortisone | 100% |
| Cutaneous use | Cream | ketoconazole | 100% |
| Cutaneous use | Cream | fluocinolone acetonide | 100% |
| Oromucosal use | Ointment | triamcinolone acetonide | 100% |
| Oral use | Suspension | alumina and magnesia | 100% |
| Gingival use | Liquid | benzidamine hidrocloride | 100% |
| Auricular use | Drops | sodium docusate | 100% |
| Vaginal use | Insert Tablet | nystatine | 100% |

detection method resulted in a sensitivity value of 100% for the nine pharmaceuticals preparations tested. This LOD value can be used as a reference by the testing laboratory in the validation or verification process of the *S. aureus* detection method for each pharmaceutical preparations.

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