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ESTUDO DE RECUPERAÇÃO DE MÉTODOS DE AMOSTRAGEM MICROBIOLÓGICA PARA SUPORTE À VALIDAÇÃO DE LIMPEZA NA INDÚSTRIA FARMACÊUTICA

RECOVERY STUDY OF MICROBIOLOGICAL SAMPLING METHODS TO SUPPORT CLEANING VALIDATION IN THE PHARMACEUTICAL INDUSTRY

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RESUMO

Introdução: Na indústria farmacêutica, a validação de limpeza é um componente crítico de um sistema eficaz de garantia da qualidade para assegurar a conformidade com os requisitos das BPF. FDA e EMA possuem diretrizes para realizar a validação de limpeza, prevenir a contaminação cruzada e minimizar o risco à segurança do paciente, mas não há orientação regulatória relacionada aos aspectos microbiológicos da validação de limpeza, técnicas de amostragem e estabelecimento de limites de contaminação microbiana para instalações farmacêuticas. Portanto, existe certo vácuo de regulamentações e princípios básicos, especialmente metodologias para conduzir adequadamente a validação de limpeza com foco na contaminação microbiológica. **Objetivo:** O presente trabalho é dedicado ao desenvolvimento e validação de métodos de amostragem por swab e enxágue de biocarga em superfícies de equipamentos de fabricação em combinação com métodos de testes microbiológicos, para demonstrar uma metodologia adequada a fim de realizar a validação de limpeza em um nível apropriado em conformidade com os requisitos das BPF. **Métodos:** Métodos de amostragem por swab e enxágue foram utilizados neste estudo. O procedimento de swab envolveu umedecer o swab (10 cm) com solução salina estéril e amostrar a área em um padrão em zigue-zague sobreposto; A amostragem por enxágue é realizada com um volume fixo de água estéril para injeção em uma superfície do equipamento. Cupons devidamente limpos e esterilizados (100 cm²) de três materiais diferentes (plástico, vidro e aço inoxidável) foram utilizados e contaminados (inoculados) com volume fixo da solução de inóculo preparada pelas suspensões de culturas bacterianas e fúngicas de trabalho. Após secar a superfície, foi realizada a amostragem por swab e enxágue para obter as amostras de teste. **Resultados e Discussão:** Métodos de amostragem por swab e enxágue foram desenvolvidos utilizando cinco culturas bacterianas e fúngicas e três diferentes tipos de materiais para obter uma boa recuperação (≥37%) com alta precisão (DPR≤15%). As taxas de recuperação para todos os tipos de materiais usando o método swab variaram de 44 a 70%, enquanto o método de enxágue foi ligeiramente inferior, variando de 35 a 57%. Dependendo do tipo de material do cupom, os resultados de recuperação dos microrganismos teste são diferentes. A tendência decrescente foi revelada na seguinte ordem: plástico, aço inoxidável e vidro. **Conclusões:** A maior recuperação foi obtida no caso de *E. coli* para ambos os métodos de amostragem. A taxa de recuperação para cada microrganismo teste é maior para o método de amostragem por swab (61%) em comparação com o método de enxágue (47%). A metodologia proposta pode ser usada para controlar a biocarga nas superfícies dos equipamentos farmacêuticos durante o processo de monitoramento microbiológico e realizar com sucesso a validação de limpeza.

Palavras-chave: *Recuperação, Amostragem por Swab e Enxágue, Validação de Limpeza, Biocarga.*

ABSTRACT

Background: In the pharmaceutical industry, cleaning validation is a critical component of an effective quality assurance system to ensure compliance with the GMP requirements. FDA and EMA have guidance to perform cleaning validation, prevent cross-contamination and minimize patient safety risk but there is no regulatory guidance relating to microbiological aspects of cleaning validation, sampling techniques and establishing microbial

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contamination limits for pharmaceutical facilities. Therefore, there is a certain vacuum of regulations and basic principles, especially, methodologies to properly conduct cleaning validation focusing on microbiological contamination. **Aim:** This present work is dedicated to the development and validation of swab and rinse sampling methods of bioburden on manufacturing equipment surfaces in combination with microbiological testing methods, to demonstrate a suitable methodology in order to perform cleaning validation at an appropriate level in compliance with the GMP requirements. **Methods:** Swab and rinse sampling methods were used in this study. The swabbing procedure involved moistening the swab (10 cm) with the sterile saline and swabbing the area to be sampled in an overlapping zigzag pattern; Rinse sampling is performed with a fixed volume of sterile water for injection from a piece of equipment surface. Properly cleaned and sterilized coupons (100 cm^2) of three different materials (plastic, glass, and stainless steel) were used and spiked (inoculated) with the fixed volume of the inoculum's solution prepared by the working bacterial and fungi culture suspensions. After drying the surface, the swab and rinse sampling was performed to obtain test samples. **Results and Discussion:** Swab and rinse sampling methods were developed using five bacterial and fungal cultures and three different types of materials in order to obtain a good recovery (≥37%) with high precision (RSD≤15%). The recovery rates for all types of materials using the swab method ranged from 44 to 70%, while the rinse method was slightly lower, ranging from 35 to 57%. Depending on the type of coupon material, the recovery results of the test microorganisms are different. The decreasing tendency was revealed in the following order: plastic, stainless steel, and glass. **Conclusions:** The highest recovery was obtained in the case of *E. coli* for both sampling methods. The recovery rate for each test microorganism is higher for the swab sampling method (61 %) compared to the rinsing method (47 %). The proposed methodology can be used to control bioburden on the pharmaceutical equipment surfaces during microbiological monitoring process and successfully perform cleaning validation.

Keywords: *Recovery, Swab and Rinse Sampling, Cleaning Validation, Bioburden*

რეზიუმე

შესავალი: ფარმაცევტულ ინდუსტრიაში, დასუფთავების ვალიდაცია წარმოადგენს GMP-ის მოთხოვნების უზრუნველსაყოფად ეფექტური ხარისხის უზრუნველყოფის სისტემის კრიტიკულ კომპონენტს. FDA და EMA გვთავაზობს გზამკვლევს დასუფთავების ვალიდაციის ჩასატარებლად, ჯვარედინი კონტამინაციის პრევენციისა და პაციენტის უსაფრთხოების რისკის შემცირებისთვის, მაგრამ არაფერს გვეუბნება მარეგულირებელი გზამკვლევი დასუფთავების ვალიდაციის მიკრობიოლოგიური ასპექტებზე, სინჯის აღების ტექნიკებსა და მიკრბიოლოგიური კონტამინაციის ზღვრების დადგენაზე ფარმაცევტულ საწარმოში. შესაბამისად, არის რეგულაციებისა და ძირითადი პრინციპების გარკვეული ვაკუუმი, კერძოდ, მეთოდოლოგიებისა, რომ სათანადოდ ჩატარდეს მიკრობიოლოგიურ დაბიძნურებაზე ფოკუსირებული დასუფთავების ვალიდაცია. **მიზანი:** წინამდებარე ნაშრომი ეძღვნება საწარმოო დანადგარების ზედაპირებზე მიკრობიოლოგიური დაბიძნურების ნაცხებისა და ჩამორეცხვის სინჯების აღების მეთოდების შემუშავებას და ვალიდაციას მიკრობიოლოგიური ანალიზის მეთოდებთან ერთად, რათა ნაჩვენები იქნას სათანადოდ მეთოდოლოგია, რათა განხორციელდეს დასუფთავების ვალიდაცია შესაბამის დონეზე GMP მოთხოვნების შესაბამისად. **მეთოდები:** ამ კვლევაში გამოყენებული იყო ნაცხებისა და ჩამორეცხვის სინჯის აღების მეთოდები. ნაცხების აღების პროცედურა მოიცავდა სტერილურ ფიზიოლოგიურ ხსნარში ტამპონიანი ჩხირის (10 სმ) დასველებას და ზიგზაგისებური გადაფარვით ზედაპირიდან სინჯის აღებას; ჩამორეცხვის სინჯის აღება ხდება სტერილური საინექციო წყლის ფიქსირებული მოცულობით დანადგარის ზედაპირიდან. გამოყენებული იქნა სამი სხვადასხვა მასალისაგან (პლასტმასი, მინა და უჟანგავი ფოლადი) დამზადებული სათანადოდ გაწმენდილი და სტერილური კუპონები (100 $სმ^2$) და მასზე დატანილი (ინოკულირებული) იქნა ბაქტერიული და სოკოების კულტურების სუსპენზიებით მომზადებული ინოკულუმის ხსნარის ფიქსირებული მოცულობა. ზედაპირის გაშრობის შემდეგ, ტამპონიანი ჩხირების გამოყენებით ნაცხებისა და ჩამორეცხვის მეთოდების საშუალებით განხორციელდა სინჯის აღება საკვლევი ნიმუშების მისაღებად. **შედეგები და განსჯა:** ნაცხებისა და ჩამორეცხვის სინჯების აღების მეთოდები შემუშავებული იყო ხუთი ბაქტერიული და სოკოვანი კულტურისა და სამი სხვადასხვა ტიპის მასალის გამოყენებით, რათა მიღებული ყოფილიყო კარგი აღდგენა (≥37%) მაღალი სიზუსტით (RSD≤15%). ყველა სახის მასალის აღდგენის მაჩვენებლები ნაცხის მეთოდით მერყეობდა 44-დან 70%-მდე, ხოლო ჩამორეცხვის მეთოდის შემთხვევაში ოდნავ დაბალი იყო და მერყეობდა 35-დან 57%-მდე. კუპონის მასალის ტიპის მიხედვით, საკვლევი მიკროორგანიზმების აღდგენის შედეგები განსხვავებულია. გამოვლინდა შემცირების ტენდენცია შემდეგი თანმიმდევრობით: პლასტმასი, უჟანგავი ფოლადი და მინა. **დასკვნები:** ყველაზე მაღალი აღდგენა მიღწეული იქნა *E. coli*-ის შემთხვევაში სინჯის აღების ორივე მეთოდისთვის. თითოეული საკვლევი მიკროორგანიზმის აღდგენის მაჩვენებელი უფრო მაღალია ნაცხის აღების მეთოდისთვის (61%) ჩამორეცხვის მეთოდთან შედარებით (47%). შემოთავაზებული მეთოდოლოგია შეიძლება გამოყენებულ იქნას მიკრობული

დაბინძურების კონტროლისთვის ფარმაცევტული დანადგარების ზედაპირებზე მიკრობიოლოგიური მონიტორინგის პროცესში და დასუფთავების ვალიდაციის წარმატებული განხორციელებისთვის.

საკვანძო სიტყვები: *აღდგენა, სინჯის აღება, დასუფთავების ვალიდაცია, მიკრობული დაბინძურება*

1. INTRODUCTION:

In the pharmaceutical industry, cleaning validation is a critical component of an effective quality assurance system to ensure compliance with the requirements of good manufacturing practice (GMP) and has the largest opportunity to prevent patient safety risk by assuring that there is no cross-contamination/contamination of drug products with variety substances such as contaminants associated with other active ingredients, cleaning and biocide agents, airborne materials, and bioburden as well (Rubashvili, *et al*., 2018; Rubashvili, *et al*., 2020; Rubashvili, 2022; Rubashvili *et al*., 2015). Cleaning with disinfection is assessed based on the level of residues that remain, either those directly found on the equipment or those indirectly contained within the final rinse after the water has passed through or over the equipment. In practice, the primary focus of cleaning validation is the removal of chemical residues, either from active ingredients or cleaning/biocide agents, and microbiological issues are of an incidental nature. It should be understood that the purpose of cleaning procedures should never be seen as being used to reduce microbial residues to acceptable levels. Many pharmaceutical companies have mistakenly claimed this in cleaning validation policies and protocols. Logically, if microorganism residues on equipment were at an unacceptable level prior to cleaning, then this implies that the batch just made must have been contaminated (Walsh, 2011; Rubashvili, 2022). Both the Food and Drug Administration (FDA) and European Medicines Agency (EMA), together with Pharmaceutical Inspection Co-operation Scheme (PIC/S), have guidance in place for cleaning validation. These are designed to prevent cross-contamination, to ensure product quality is maintained, and to minimize patient safety risk (FDA Inspection guide 7/93. 2014; EU Guidelines for Good Manufacturing Practice for Medicinal Products for Human and Veterinary Use. 2015; PIC/S PI 006-3. 2007). There is no regulatory guidance relating to microbiological aspects of cleaning validation. There are no general principles for sampling and establishing microbial contamination limits to validate cleaning and disinfection procedures in pharmaceutical facilities. These regulations provide only sampling techniques and related requirements, along with general principles for selecting products that may pose a risk of cross-contamination or represent worst-case scenarios for cleaning procedures. They also outline rules for establishing acceptable limits for chemical residues to prevent cross-contamination in shared pharmaceutical facilities. However, there are no specific requirements for microbiological contamination or for selecting products with a worst-case scenario for microbial contamination to be cleaned. Therefore, there is a certain vacuum of regulations and basic principles, especially, methodologies to properly conduct cleaning validation focusing on microbiological contamination (Rubashvili, *et al*., 2018; Rubashvili, *et al*., 2020; Rubashvili, 2022; Rubashvili *et al*., 2015). The United States Pharmacopeia outlines key aspects of microbiological contamination monitoring, including a requirement to evaluate the recovery of sampling methods used to control such contamination. However, it does not provide more specific or technical details (USP General monograph <1116>. 2024).

A basic literature review revealed that various online resources discuss the microbiological aspects of cleaning validation, offering methodological guidelines, standard procedures, and protocols. However, the scientific literature lacks in-depth research and detailed descriptions in the form of methodology. Most publications focus on the development of active ingredient residue methods, validation, assessment of cross-contamination risks, sampling, recovery studies of sampling methods, establishment of acceptable limits, and the execution of cleaning validation (Lakshmana Prabu *et al*., 2015; Dyer et al, 2012; Raj Pal Govind *et al*., 2018).

Cleaning validation is a multi-step and sequential process. One of the critical stages is the analytical part, which ultimately determines the successful implementation of cleaning validation. The analytical part of cleaning validation includes the following activities: selection of the "worst case" drug product, identification of product direct contact surfaces of shared manufacturing

equipment, determination of the areas of identified surfaces, identification of the most difficult-to-clean points on the surfaces according to their geometric shapes, accessibility for cleaning and disinfection, development and validation of sampling methods for residues, including bioburden, as well as validation of specific and non-specific analytical methods for quantitative estimation of residues. Direct and indirect sampling methods should be developed depending on the material, geometrical shape, and cleaning accessibility of the equipment surfaces. A sound microbiological sampling plan is required to evaluate microbiological risks. The emphasis on sampling is important since microorganisms cannot be introduced into the process. This is unlike a chemical assessment where equipment can be deliberately soiled with a residue to test out cleaning efficacy (Sandle, 2017; Rubashvili, *et al*., 2020; Rubashvili *et al*., 2015).

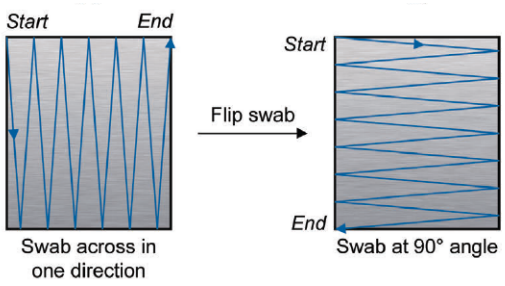
This present work is dedicated to the study of the important analytical part of cleaning validation, including the development and validation of sampling methods of bioburden on manufacturing equipment surfaces in combination with microbiological testing methods, to demonstrate a suitable methodology in order to perform cleaning validation at an appropriate level in compliance with GMP requirements and confirm that pharmaceutical formulations produced on shared pharmaceutical facilities are free from the risk of microbiological contamination. This issue is crucial for pharmaceutical manufacturers, as the adequacy and suitability of the selected sampling techniques must be confirmed to carry out cleaning validation and microbiological monitoring of production premises and equipment. Specifically, it must be ensured that the sampling methods can effectively take and then test the bioburden with high recovery rates, as accurate and reliable results in environmental microbiological control depend on this. The proposed methodology offers a valuable opportunity to address a methodological gap that is often inadequately implemented in the quality assurance programs of the pharmaceutical industry. The paper describes a recovery study for direct and indirect sampling methods, combined with microbiological enumeration testing, for bioburden determination on shared manufacturing equipment surfaces in support of cleaning validation. The recoveries of various pathogenic microbes are determined using both methods on the surfaces of different materials, along with the influence of various factors on the recovery rate.

2. MATERIALS AND METHODS:

2.1. Materials and Methods

Swab and rinse sampling methods available to conduct cleaning validation were used in this study. The swabbing is a direct method and a subjective manual procedure that involves physical interaction between the swab and the equipment surface and varies from sampler to sampler. The surface was successively wiped with one sterile swab moistened with the sterile saline solution. The scheme of the swabbing procedure is shown in Figure 1 (The ISPE Guide: Cleaning Validation Lifecycle – Applications, Methods, and Controls. 2020). The swabs (10 cm, small woven, polyester, Copan, Italy) were placed in the screw-cap test tubes containing 10 mL of sterile saline. The swabbing procedure involved moistening the swab with the sterile saline and swabbing the area to be sampled in an overlapping zigzag pattern; first, the surface area was wiped vertically from one side to the other (up and down), then, after rotating the swab, horizontally (back and forth). A fresh surface was exposed and repeatedly wiped to extract the maximum bioburden. Finally, the swab was secured in closed and labeled tubes for bioburden control using microbiological enumeration testing methods (Rubashvili, *et al*., 2018; Rubashvili, *et al*., 2020; Rubashvili, 2022).

Figure 1. *The scheme of swabbing technique*



Rinse sampling is an example of indirect sampling, as any remaining surface residue is not taken directly from the equipment surface. A separate sampling performed it rinse with a fixed volume of sterile water for injection (WFI) from a piece of equipment (Rubashvili, *et al*., 2018).

Properly cleaned and sterilized coupons, which are pieces of three different materials (plastic, glass, and stainless steel) representing pharmaceutical manufacturing equipment to be sampled, were used. A coupon typically 10×10 cm (100 cm^2) was representative of a standard sample size for smaller irregular surfaces and larger flat surfaces. A commercial disinfectant-detergent (Microbac Forte, Bide, Germany) containing quaternary ammonium salts (0.25 %

solution) and sterile 70 % isopropanol (analytical grade, Merk Millipore, Germany) is used for cleaning and disinfection of selected coupons.

The preparation procedures related to test microorganisms, test samples and coupons were carried out in a GMP A grade cleanroom condition, such as within a biosafety cabinet class II with laminar airflow (Thermo Fischer Scientific, USA). For spiking (inoculation) of challenge microorganisms on test coupons, the working bacterial and fungi cultures were prepared. These cultures included a Gram-negative rod, Gram-positive cocci, yeast, mold, and spore former microorganisms - *Staphylococcus aureus ATCC 6538, Pseudomonas aeruginosa ATCC 9027; Candida albicans ATCC 2091; Escherichia coli ATCC 8739, Bacillus subtilis ATCC 6633.* The cultures were reconstituted according to the vendor's instructions (American Type Culture Collection, USA). After the specified incubation period, the bacteria and fungi cultures were harvested by washing each plate with 2 mL of sterile saline, then the culture liquid media was centrifuged until a microbial pellet formed at the bottom of the test tube; then the obtained supernatant was removed and the microbial pellet resuspended in sterile saline.

The bacterial suspensions were adjusted to a value absorption of 0.2 by sterile buffer diluent - 0.05 % polysorbate 80 solution (high purity Eur. Ph. Grade, Merck Millipore, Germany) using a UV-Vis spectrophotometer – Shimadzu UV-1800 (Japan) at 550 nm. The fungi suspensions were also adjusted to 5.0 McF standard by buffer diluent using a Biosan Den-1 densitometer (Latvia). Using a standard serial dilution method, the inoculum solution of each challenge microorganism was prepared in the sterile saline solution from the prepared suspensions. Each type of coupon surface was inoculated (spiked) with 100 µL (~100 CFU) of the inoculum's solution using a micropipette so as to spread the solution onto the coupon. The surface of the spiked coupon was allowed to dry for a contact time of less than 30 min. After drying the surface, the swab sampling was performed according to the swab procedure. The swab sampling procedure was performed in triplicate (n=3) for each challenge microorganism. The swab sample was diluted with the saline to 10 mL in the labeled tube. Subsequently, the tubes were placed on a vortex mixer (China) for 1-2 minutes. The obtained swab samples were tested using the plate-count method (USP General monograph <61>. 2024). The swab aseptically was removed, and 100 µL of the swab sample was inoculated onto a tryptic soy agar (TSA) plate with lecithin and polysorbate 80 (Merk Milipore, Germany) for bacteria and sabouraud dextrose agar (SDA) (Merk Milipore, Germany) plate for fungi with an incubation condition at 35ºC for three days for bacteria and 25 ºC for five days for fungi.

The incubation of test samples was performed in a laboratory thermostat-incubator (Thermo Fischer Scientific, USA).

Additionally, blank, swab negative, and positive control samples were prepared in duplicate (n=2). The swab-negative control sample was prepared by placing a sterile swab into a tube and then diluted to 10 mL with sterile saline. The swab-positive control sample was prepared by placing an unused sterile swab into a sterile tube, adding 100 µL of each challenge microorganism inoculum's solution containing 100 colony-forming units (CFU), and then diluting to 10 mL with sterile saline. To assess the antimicrobial activity of the swab and the compatibility of the swab material, additional positive control samples were prepared for each test microorganism in the same manner but without the swab. For the preparation of the blank, the untreated coupon was wiped with a wetted swab with sterile saline. The swab was placed into a tube and then diluted to 10 mL with the same diluent. After incubation, the number of CFU per plate was enumerated, and the average number of CFU and the recovery rate % for each test microorganism were calculated.

For rinse sampling, using sterile forceps, aseptically, the surface area of the inoculated coupon (~10^3 CFU) was rinsed with approximately 100 mL of the sterile WFI in a sterile beaker and then transferred and diluted to volume with the same diluent to 100 mL in a sterile flask, mixed well. The obtained rinse samples were tested using the membrane filtration method. The flask was placed onto a shaker for 5 min, and the diluent was placed through a 0.45 µm membrane filter using the vacuum filtration system. Aseptically, the used filter was removed and placed onto a TSA plate. The plate was incubated at the same conditions. The blank, positive, and negative control samples were prepared similarly to the swab method recovery study. This procedure was performed in triplicate as well. The scheme of sample preparation using both sampling methods are shown in Figure 2.

All the measuring equipment were appropriately calibrated and qualified. The experiment was carried out in controlled laboratory area (temperature, t= 22±3^{0}C, relative humidity, RH>65%).

2.2. Calculations

The relative difference (Diff, %) was used to evaluate the antimicrobial activity of the swab and the compatibility of the swab material. This value was calculated for each test microorganism as the percentage difference between the number of colonies enumerated on plates of the positive control sample and the positive control sample without the swab.

The average recovery (R_i, %) of the combination of sampling and microbiological enumeration test methods for each challenge microorganism was calculated by Equation 1:

$$R_i, \% = \frac{CFU(n)_R}{CFU(n)_A} \times 100 \quad \text{(Eq. 1)}$$

Where, $CFU(n)_R$ is the average number of recovered colonies (recovered amount – from the test sample's Petri dishes, n=3), and $CFU(n)_A$ is the average number of inoculated colonies (amount added – from the positive control sample's Petri dishes, n=2).

The average recovery (R_{iAv}, %) for each challenge microorganism for all types materials was calculated by Equation 2:

$$R_{iAv}, \% = \frac{\sum_i^z R_i}{z} \quad \text{(Eq. 2)}$$

Where, z is the number of types of materials.

The percentage mean recovery (R, %) was calculated by Equation 3:

$$R, \% = \frac{\sum_i^n R_{iAv}}{5} \quad \text{(Eq. 3)}$$

Where, 5 is the number of test microorganisms.

A confidential interval (95%CI) at the 95% confidence level for each test sample result was calculated by Equation 4:

$$95\% CI = t_{0.05,n-1} \times \frac{SD}{\sqrt{n}} \quad \text{(Eq. 4)}$$

Where, t is critical value of student's distribution for one-tailed test at significance level α=0.05; n is the number of replicates (n=3) of the test samples; SD - standard deviation of replicates (n=3) of the test samples.

An average number of CFUs counted on the each Petri dish (CFU(n)) was calculated by Equation 5:

$$CFU(n) = \frac{\sum_{i=1}^n CFU_i}{N} \quad \text{(Eq. 5)}$$

Where, CFU(n) is the number of CFU counted on each Petri dish; N is the quantity of petri dishes.

3. RESULTS AND DISCUSSION:

3.1. Results

The swab and rinse sampling procedures were developed in order to obtain a suitable and good recovery for each challenge microorganism and each type of equipment material. In order to increase and optimize the recovery rate for both sampling methods, the influence of the material of the coupon, the material and type of the swab and the tube for the sample, the swabbing technique, the contact time with the coupon material, the sampling area, the nature and volume of the solvent, the volume and size of the inoculum's solution, and the incubation time and temperature were investigated. In order to evaluate the influence of critical factors on the recovery rate and establish optimal parameters for sampling methods, which are fully described above.

Recovery studies were conducted using all five bacterial and fungal test cultures on all three types of material coupons. Swab and rinse test samples were prepared in triplicate, as well as positive control, negative control, and blank samples in duplicate. The recovery rates (R_i, %) for each challenge microorganism, the average recovery rates (R_{iAV}, %) for each challenge microorganism and the mean recoveries (R, %) for both sampling methods, as the relative standard deviation (RSD) of the recovery rates for each test sample (triplicates, n=3) were calculated. The RSD was used to assess the variation within samples and the precision rate of triplicates of each test sample. An acceptance limit (AL) for each recovery rate including the mean recovery (R, %) is ≥35 %, and a recommendation limit for the RSD - ≤20 %. The Diff, % was used for evaluation of the compatibility of swab material to demonstrate that this material does not retain test microorganisms and does not have an antimicrobial effect on them. The acceptance limit was considered 30%, and less than this value means that an antimicrobial effect is not observed and test microorganisms absorbed by the swab

material are completely transferred into the solution to be plated for incubation.

The enumerated colonies obtained with test samples on three types of material coupons, also positive control, positive control without swab, negative control samples and blank are given in Table 1, 2. In the case of the swab sampling method, the highest number of colonies was enumerated on the plates obtained with plastic material, and the lowest number was observed on the plates obtained with glass material. The highest growth was appeared in the case of *E. coli*, namely, the number of CFU was 39 on plastic material, 34 on glass material, and 36 on stainless steel material. The lowest growth was observed in the case of C. *albicans*, namely, the number of CFU was 21 was on plastic material, 17 on glass material, and 19 on stainless steel material. The same results were obtained using the rinse sampling method, although slightly different, with the number of colonies of all microorganisms being slightly lower on the plates of samples taken from all types of materials. For example, on plastic, glass and stainless steel materials, the number of colonies of *P. aeruginosa* was 29, 19, 25, respectively. The results of the blank and negative control samples show that no growth was observed. The Diff, % between the numbers of colonies enumerated on plates of the positive control sample and the positive control sample without the swab for each test microorganism were significantly less the limit (30%). The largest value of this difference was observed in the case of *S. aureus*, equal to 9%.

The calculated values of the mean recovery - R, %, the average recovery - R_{iAv}, %, the recovery - $R_{i,}$ % and the RSD % for each test microorganism are depicted as tables and diagrams (Table 1, 2 and Figure 3, 4). The results obtained show that for all test microorganisms, the recovery rate was highest on plastic material and lowest on glass material. For example, for *S. aureus,* when using the swab method, the R_i, % on plastic material was 66%, on stainless steel - 44%, and on glass - 39%. The recovery rates for all types of materials using the swab method ranged from 44 to 70%, while the rinse method was slightly lower, ranging from 35 to 57%. The highest recovery was observed for *E. coli* on plastic material for both swab and rinsing sampling methods and equal to 70%, and 57%, respectively. There was a slight difference in the average recovery rates for all microorganisms on all three types of materials, with the swabbing method having a higher recovery rate than the rinsing method. The highest average recovery was observed when using both methods, for all types of materials, in the case of *E. Coli* and equal to 65% and 52%, respectively, while the lowest was in the case of *S. aureus*, 57% and 43%, respectively. The mean recovery was 61% for the swab sampling method and 47% for the rinse sampling method, although all results were above the acceptable criteria. The obtained results also show that the variation between triplicates within each test sample was not more and did not exceed the recommended limit, in particular, the highest RSD value was observed in the case of *B. subtilis* and equal to 15%, when using the swabbing method, while when using the rinsing method, the highest value was observed in the case of *P. aeruginosa*, also equal to 15%.

3.2. Discussion

Depending on the type of coupon material, the recovery results of the test microorganisms are different. The decreasing tendency was revealed in the following order: plastic, stainless steel, and glass. This tendency is typical for both sampling methods. The lowest recovery was observed for glass, which is a smooth, non-porous, inert material. In dry conditions, the tendency of viable bacteria to die is observed. However, better recovery results were obtained on the stainless steel surface. It is also a non-porous, smooth, inert material, but it shows increased adherence to microorganisms. All the good recoveries were obtained on plastic material, which is a porous inert material; therefore, the adherence ability is increased, and the death of microorganisms is less. The plastic can absorb the bacteria adherent to the surface and improve the recovery result. The recovery rates for different microorganisms on the same material are not significantly different. Regardless of the nature of the material and performance of sampling technique, for both sampling methods used for bioburden testing on pharmaceutical manufacturing surface, the highest recovery was observed in case of *E. coli*, and the lowest in case of *S. aureus*. This fact clearly demonstrates the good adherence ability of *E. coli* to surfaces and its viability compared to other test microorganisms. The mean recovery is much more than the acceptance limit for both sampling methods, which confirms the validity and appropriateness of the combination of sampling and microbiological testing methods. The calculated values of RSD of the recovery rates obtained (triplicates, n=3) from the test samples are below the recommended limit (≤20%), which confirms that both sampling methods are characterized by high recovery and less precision

despite the complexity of its execution technique.

The absence of growth in the blank and negative control samples confirmed that the environment and materials used are free from microbiological contamination and the recovery studies were performed under aseptic conditions, and glassware, solvents, swabs, media, and coupons were sterile.

4. CONCLUSIONS:

Hence, swab and rinse sampling methods for microbiological contamination testing on surfaces of pharmaceutical equipment were developed in order to obtain a good recovery (≥37%) with high precision (RSD≤15%). The recovery studies were performed using five bacterial and fungal cultures and three different types of materials. The highest recovery was obtained in the case of *E. coli* for both sampling methods. The recovery rate for each test microorganism is higher for the swab sampling method (61 %) compared to the rinsing method (47 %). The proposed methodology can be used to control bioburden on the pharmaceutical equipment surfaces during microbiological monitoring process and successfully perform cleaning validation.

5. DECLARATIONS

5.1. Study Limitations

Several limitations should be considered when interpreting and applying the results of this study. The methodological scope was constrained to three specific surface materials (plastic, glass, and stainless steel), which may not fully represent the diversity of materials and surface finishes encountered in pharmaceutical manufacturing equipment. The experimental design utilized a relatively small sample size with three replicates for test samples and two for controls, potentially affecting the statistical robustness of our findings. Technical constraints included the use of standardized inoculum volumes (100 µL) and concentrations (~100 CFU), which may not reflect the variable contamination levels encountered in real manufacturing environments. Additionally, the brief contact time between inoculation and sampling (less than 30 minutes) may not adequately represent conditions where contaminants persist for extended periods on equipment surfaces. Furthermore, the selected panel of five microorganism species, though representative of major microbial groups, may not encompass the full spectrum of potential contaminants, particularly in mixed-population scenarios. The validation approach could be strengthened through interlaboratory testing to confirm method reproducibility across different facilities and operators. Future studies should consider expanding the scope to address these limitations and provide more comprehensive validation of these sampling methods under diverse real-world conditions.

5.2. Funding source

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5.3. Competing Interests

The authors declare that they have no conflicts of interest regarding the publication of this article.

7. REFERENCES:

1. Govind, R. P., Arya Rajeshwar Kamal Kant, A. R. K., Tanuj, J., Dheeraj, B. A (2018). Review on cleaning validation in pharmaceutical industry. *Journal of Drug Delivery and Therapeutics*. 8 (3), 138-146. DOI: 10.22270/jddt.v8i3.1695
2. Dyer, R. L., Frank, J. F., Johnson, B., Hickey, P., Fitts, J. (2004). Chapter 13 - Microbiological Tests for Equipment, Containers, Water and Air. *Standard Methods for the Examination of Dairy Products*, Ed. 17th ed. American Public Health Association, Washington, USA, 328
3. EudraLex Volume 4. EU Guidelines for Good Manufacturing Practice for Medicinal Products for Human and Veterinary Use. Annex 15: Qualification and Validation. 2015
4. FDA Guide to Inspections Validation of Cleaning Processes. 7/93. 2014
5. Lakshmana Prabu, S., Suriya Prakash, T. N. K., Thirumurugan, R. (2015). Chapter 5 - Cleaning Validation and Its Regulatory Aspects in the Pharmaceutical Industry. *Developments in Surface Contamination and Cleaning,* 129-186. DOI: 10.1016/B978-0-323-31303-2.00005-4
6. PIC/S Validation-Master Plan, IQ, OQ, non-sterile Process Validation, Cleaning Validation (PI 006-3). 2007
7. Rubashvili, I., Karukhnishvili, N., Makharadze N. (2018). Vincamine residues analysis using HPLC and establishing limits of cross-contamination in support of cleaning validation. *Revue Roumaine de Chimie*, 63(3), 205-215.
8. Rubashvili, I., Karukhnishvili, N., Makharadze, K. (2020). Simultaneous quantitative estimation of lisinopril and hydrochlorothiazide residues using HPLC for cleaning validation. *Chemistry Journal of Moldova,* 15(1), 8-20. DOI: 10.19261/CJM.2020.669
9. Rubashvili, I. (2022). Quantitative determination of terbinafine residues on the pharmaceutical manufacturing equipment surfaces. *Periodico Tchê Química*, 19(40), 26-34. DOI: 10.52571/PTQ.v19.n40.2022.03_RUBASHVILI_pgs_26_34.pdf
10. Rubashvili, I., Karukhnishvili, N., Loria, K., Dvali, N. (2015). Validation of swab sampling and HPLC methods for determination of meloxicam residues on surfaces of pharmaceutical manufacturing equipment surfaces for cleaning validation. *Turkish Journal of Pharmaceutical Sciences,* 12(3), 287-298. DOI: 10.5505/tjps.2015.43531
11. Sandle, T. J. (2017). Microbiological Aspects of Cleaning Validation. *GxP Compliance*, 21 (5), 1
12. Walsh, A. (2011) Microbial aspects in cleaning validation. In Saghee, M. R, Sandel, T. and Tidswell, E. C. (Eds.) Microbiology and Sterility Assurance in Pharmaceutical Industry, Business Horizons, New Dehli, 1-14
13. The ISPE Guide: Cleaning Validation Lifecycle – Applications, Methods, and Controls. 2020
14. The United States Pharmacopeia. General chapter <1116>. Microbiological control and monitoring of aseptic processing environments. 2024
15. The United States Pharmacopeia. General chapter <61>. Microbiological examination of nonsterile products: microbial enumeration tests. 2024

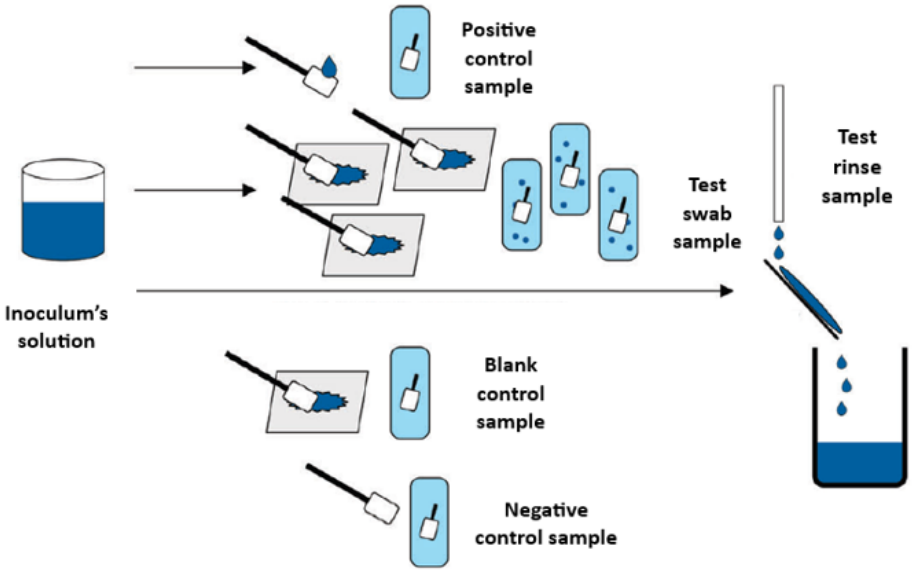

Figure 2. *Sample preparation for recovery studies of swab and rinse sampling methods*

Table 1. *The results of recovery studies for swab sampling method (CFU_R - the average number of recovered colonies; RSD,% - the relative standard deviation of the recovery rates for each test sample (n=3); R_i,% - the average recovery of each challenge microorganism; R_{iAv},% - the average recovery for each challenge microorganism for all types materials; Diff,% - the percentage difference between the numbers of colonies enumerated on plates of the positive control sample and the positive control sample without the swab)*

Sample/ Coupon material	***Value***	***Test microorganism***				
		S. aureus	***P. aeruginosa***	***C. albicans***	***E. coli***	***B. subtilis***
Test sample (n=3)/ Plastic	**$CFU(n)_R$**	27	35	21	39	32
	R_i,%	66	67	66	70	68
	RSD, %	13	11	9	7	15
	95%CI	16	15	12	9	19
Test sample (n=3)/ Glass	**$CFU(n)_R$**	18	28	17	34	23
	R_i,%	44	54	53	61	49
	RSD, %	11	10	14	13	10
	95%CI	15	13	19	18	13
Test sample (n=3)/ Stainless steel	**$CFU(n)_R$**	25	33	19	36	29
	R_i,%	61	63	59	64	62
	RSD, %	9	7	14	14	8
	95%CI	11	9	18	18	10
Positive control sample (n=2)	**$CFU(n)_A$**	41	52	32	56	47
Positive control sample without swab (n=2)	**CFU(n)**	44	54	35	59	51
Negative control sample (n=2)	-	No growth	No growth	No growth	No growth	No growth
Blank (n=2)	-	No growth	No growth	No growth	No growth	No growth
Positive control sample/positive control sample without the swab	**Diff, %**	7	4	9	5	8
-	**R_{iAv}, %**	57	62	59	65	60

Table 2. *The results of recovery studies for rinse sampling method (CFU_R - the average number of recovered colonies; RSD,% - the relative standard deviation of the recovery rates for each test sample (n=3); R_i,% - the average recovery of each challenge microorganism; R_{iAv},% - the average recovery for each challenge microorganism for all types materials; Diff,% - the percentage difference between the numbers of colonies enumerated on plates of the positive control sample and the positive control sample without the swab)*

Sample/ Coupon material	Value	Test microorganism				
		S. aureus	P. aeruginosa	C. albicans	E. coli	B. subtilis
Test sample (n=3)/ Plastic	$CFU(n)_R$	20	29	16	31	27
	R_i,%	66	67	66	70	68
	RSD, %	13	11	9	7	15
	95%CI	16	15	12	9	19
Test sample (n=3)/ Glass	$CFU(n)_R$	16	19	12	26	17
	R_i,%	44	54	53	61	49
	RSD, %	11	10	14	13	10
	95%CI	15	13	19	18	13
Test sample (n=3)/ Stainless steel	$CFU(n)_R$	17	25	15	28	25
	R_i,%	61	63	59	64	62
	RSD, %	9	7	14	14	8
	95%CI	11	9	18	18	10
Positive control sample (n=2)	$CFU(n)_A$	41	52	32	56	47
Negative control sample (n=2)	-	No growth	No growth	No growth	No growth	No growth
Blank (n=2)	-	No growth	No growth	No growth	No growth	No growth
-	R_{iAv}, %	43	47	45	52	47

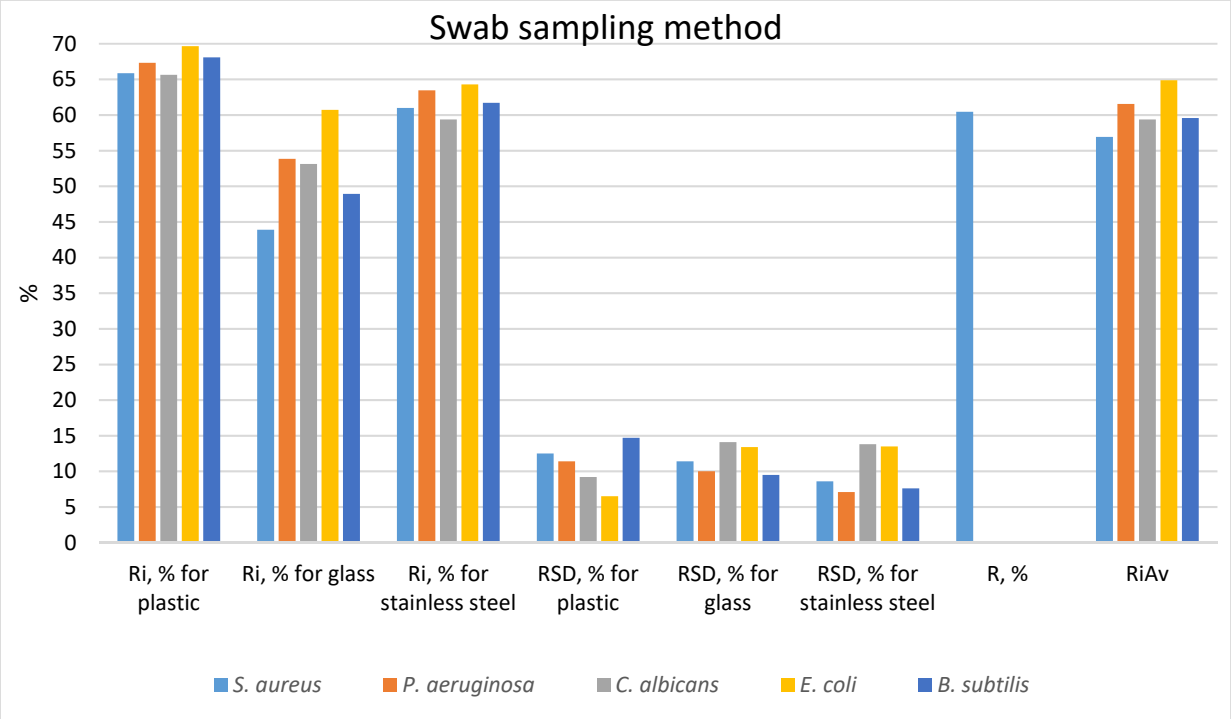

Figure 3. *Diagram of calculated values of the mean recovery (R, %), the average recovery rates (R_i) for each test microorganism, the average recovery rates (R_{iAv}) for each microorganism for all the type materials and the relative standard deviations (RSD, %) for each test microorganism for the swab sampling method*

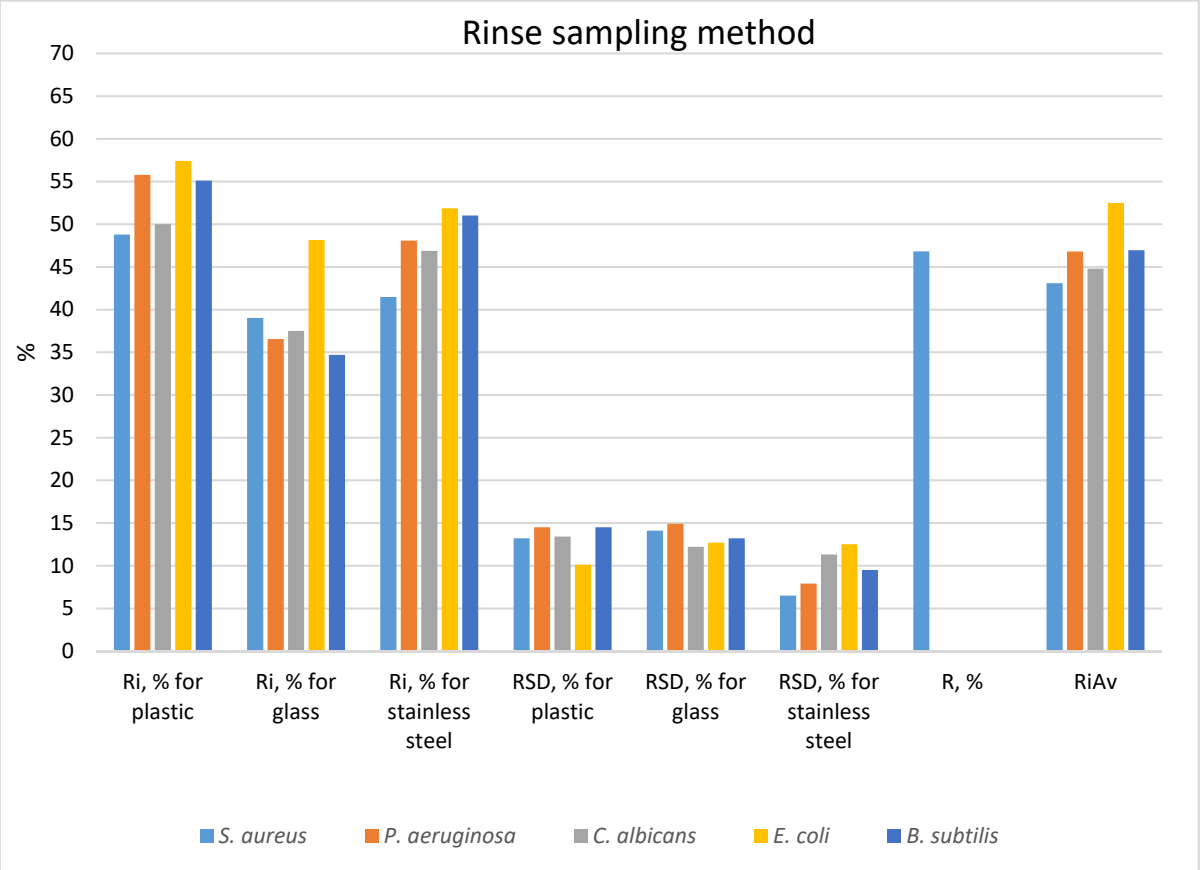

Figure 4. *Diagram of calculated values of the mean recovery (R, %), the average recovery rates (R_i) for each test microorganism, the average recovery rates (R_{iAv}) for each microorganism for all the type materials and the relative standard deviations (RSD, %) for each test microorganism for the rinsing sampling method*