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Evaluation of Bioindicator in Ampoules by Membrane Filtration

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1. Introduction

The Radiopharmacy Center of IPEN-CNEN produces a wide range of radiopharmaceuticals, some of which are manufactured under aseptic conditions with microbiological quality control of raw materials and intermediate process solutions to ensure the sterility of the final product.

Wet heat sterilization – autoclaving - is the terminal process that ensures the sterility of GAL-IPEN (gallium citrate [Ga-67]) and CARD-IPEN (thallium chloride [Tl-201]) products. Autoclaving is also the preferred method for sterilizing the sodium molybdate – Mo-99 solution before it is added to the alumina columns during the production of GERADOR IPEN-TEC. For monitoring autoclave sterilization, the additional use of biological indicators [1] is considered, which are more representative of effective elimination of viable microorganisms than chemical indicators and temperature and pressure records of the autoclave.

The biological indicator currently used in the Radiopharmacy Center is the Sterikon® plus, manufactured by Merck-Millipore. This bioindicator consists of an ampoule containing nutrient broth, sugar, pH indicator, and *Geobacillus stearothermophilus* (ATCC 7953) spores. If the sterilization process is adequate, *G. stearothermophilus* spores are killed, and the ampoule's content remains red-light to reddish-purple after incubation at 60 ± 1 °C for 48 hours. In the event of inadequate sterilization, some spores survive, and the ampoule's content changes from yellowish to yellowish-orange within 24 hours due to acid formation from the fermentation of sugars present in the nutrient broth [2].

In routine procedures, we frequently observe bioindicator ampoules with yellowish-orange content shortly after autoclaving the sodium molybdate-99Mo solution. Color changes also occasionally vary between colorless, pinkish, and brownish. The yellowish-orange color is similar to that obtained in the case of ineffective sterilization after incubation at 60 ± 1 °C for 48 hours. For this reason, it would not be possible to confirm the effectiveness of sterilization when ampoules of a color different from red-light to reddish-purple are generated. In practice, the test with biological indicators would be invalid, contradicting sanitary regulations related to the production of sterile drugs [1].

Therefore, it is necessary to develop a technique that allows the detection of viable microorganisms in the bioindicator content without relying only on color as a reference. The presence or absence of viable *G. stearothermophilus* denotes an ineffective or effective sterilization process, respectively - a fundamental result for the final release of a given batch of sterile radiopharmaceuticals.

In this study, we propose the membrane filtration technique for detecting viable *G. stearothermophilus* bacteria in the bioindicator after incubation at 60 ± 1 °C for 48 hours. The application of this technique involves, in routine situations, handling ampoules externally contaminated with radionuclides from the production process. To minimize the radiological risk to which the analyst in the Microbiological Laboratory would be exposed during ampoule manipulation, we also investigated the possibility of conducting the assay after the radioactive decay of residual radionuclides in the ampoules.

2. Methodology

(a) Test suitability – membrane filtration

The suitability was assessed using two analytical matrices: MATRIX 1, representing a bioindicator subjected to effective sterilization (light red to reddish-purple after incubation); MATRIX 2, characteristic of a bioindicator subjected to an ineffective process (yellowish to yellowish-orange after incubation). The ampoules of the Sterikon® Plus bioindicator were of batch Z0768474.

Membrane Filtration Technique – The content of an ampoule was transferred to Rinse Fluid A (0.1% meat peptone broth) using aseptic technique, following filtration (Milliflex system) and attachment of the filtration membrane to 60 mm soy-trypticase agar plate. The assembly was incubated at 60 ± 1 °C for 48 hours. After incubation, bacterial colonies were manually counted, with inspection of both sides of the filter membrane.

MATRIX 1 preparation – Two vials of the bioindicator were subjected to autoclaving at 121 °C for 20 minutes, followed by drying (approximately 80 °C) for 30 minutes. The content of the vials remained purplish after autoclaving.

G. stearothermophilus suspensions preparation – The entire content of a bioindicator vial was transferred to a 2 mL microtube and centrifuged at 43,100 g for 10 minutes at 4 °C. The supernatant was discarded and 2 mL of sterile 0.9% NaCl were added to the microtube. After one minute vortexing, the suspension was subjected to serial dilution in sterile 0.9% NaCl at dilutions of 1:10 and 1:100. The 1:100 dilution corresponded to an average concentration of 4.8×10^3 $CFU.mL^{-1}$ (CFU: colony-forming unit) and was taken as the working suspension (average of 96 CFU in 20 µL). The suspensions were kept cold until use.

MATRIX 2 preparation – Two vials of the bioindicator were incubated at 60 ± 1 °C for 48 hours. After incubation, the vials were autoclaved at 121 °C for 20 minutes, followed by drying (approximately 80 °C) for 30 minutes. The content of the vials changed from purplish to yellowish (slightly cloudy) after incubation, due to the proliferation of microorganisms. Autoclaving did not alter the color observed after incubation.

Test suitability – Both vials of MATRIX 1/2 were opened using aseptic technique, and 20 µL of sterile 0.9% NaCl were added to one of the vials of the duplicate (negative control). To the other vial (test), 20 µL of the *G. stearothermophilus* suspension were added. The entire contents of the vials were subjected to the membrane filtration technique, as described earlier. The quantitative control of the microbial suspension involved applying 20 µL of the suspension onto 80 µL of sterile saline, on duplicate 90 mm soy-trypticase agar plates, and spreading with a sterile Drigalsky loop. The plates were incubated at 60 ± 1 °C in a humid chamber for 48 hours. The mean microbial counts from the duplicate were considered as 100% in the calculation of microbial recovery during MATRIX 1/2 testing.

(b) Residual radioactivity in bioindicator ampoules

In order to reduce the radiological risk of handling contaminated vials, we proposed that the opening should take place after the decay of residual radioactivity in the vials, a condition tested in

(c). We considered samples to have decayed when they reached the exemption levels for solid materials containing radionuclides, as established in the standard ***Gerência de Rejeitos Radioativos de Baixo e Médio Níveis de Radiação*** [3]. Thus, for each radionuclide in this study (Mo-99, Ga-67 and Tl-201), the exemption levels are: an activity concentration of 1×10^2 $kBq.kg^{-1}$; activity of 1×10^6 Bq. The arithmetic mean of twenty vials masses was used in calculating the activity concentration at the end of the ampoules incubation.

Estimation of the radioactivity of ampoules at the incubation period end - Vials from the production processes of GERADOR IPEN-TEC, GAL-IPEN and CARD-IPEN were collected over six months, and their activities were determined by gamma spectrometry (Canberra system). Activity immediately after incubation (A0) calculation follows the expression:

$$A0 = \frac{A}{e^{[-(\ln 2).\Delta t]/T}} \quad (1)$$

Where A stands for activity, Δt is the elapsed time between the end of incubation and the activity measurement, and T is the physical radionuclide half-life (65.94 h for Mo-99; 3.2612 days for Ga-67; 72.912 h for Tl-201) [4]. The residual radioactivity at the end of incubation was estimated by adding the arithmetic mean to three standard deviations, corresponding to the set of activities A0 for each radionuclide – a 'worst-case scenario' with the longest decay time.

(c) Qualitative test applied to bioindicators

The combination of microbial load variables (stability, reduction or elimination) and the timing of the qualitative test (with or without decay) results in six situations, simulated as follows:

(i) High load, no decay – Vial incubated at 60 ± 1 °C for 48 hours;

(ii) High load, with decay – Vial incubated at 60 ± 1 °C for 36 days (48 hours of routine incubation, plus 34 days to simulate decay);

(iii) Load eliminated, no decay – Vial autoclaved at 121 °C for 20 minutes, with a drying period (approximately 80 °C) for 30 minutes;

(iv) Load eliminated, with decay – Vial autoclaved at 121 °C for 20 minutes, with a drying period (approximately 80 °C) for 30 minutes. Subsequently, the vial was incubated at 60 ± 1 °C for 36 days (48 hours of routine incubation, plus 34 days to simulate decay);

(v) Load reduced, no decay – Vial autoclaved at 121 °C for 04 minutes, without a drying period. Subsequently, the vial was incubated at 60 ± 1 °C for 48 hours;

(vi) Load reduced, with decay – Vial autoclaved at 121 °C for 04 minutes, without a drying period. Subsequently, the vial was incubated at 60 ± 1 °C for 36 days (48 hours of routine incubation, plus 34 days to simulate decay).

In all conditions, the entire content of the vial was subjected to the membrane filtration technique, as described in (a). The plate was incubated at 60 ± 1 °C for 48 hours.

3. Results and Discussion

Microbial recovery of *G. stearothermophilus* was calculated for the two tested matrices, assuming the mean counts in the duplicate plates of the quantitative control of the microbial suspension as 100%. For MATRIX 1 (bioindicator subjected to effective sterilization), the recovery was 236.4%, and for MATRIX 2 (bioindicator subjected to ineffective sterilization), it reached 105.6%. Therefore, the recoveries were satisfactory (greater than or equal to 50%) in both matrices, indicating that the qualitative test with membrane filtration is suitable for the intended use.

The decay time until the exemption limit was 31 days for Mo-99 vials, sixteen days for Tl-201 vials, and 13 days for Ga-67 vials. The calculated decay time for Mo-99, being the longest, was taken as a reference for the qualitative test applied to the bioindicators. The results of this test are described as follows: (i) high load, no decay: over 100 CFU; (ii) high load, with decay: colonies too numerous to count (over 100 CFU); (iii) load eliminated, no decay: absence of colonies; (iv) load eliminated, with decay: absence of colonies; (v) reduced load, no decay: 49 CFU; (vi) reduced load, with decay: absence of colonies.

The decay period did not cause a significant reduction in microbial load in the non-autoclaved vials (high load), unlike what occurred in the reduced load test. The reduced population of microorganisms survived incubation at 60 ± 1 °C for 48 hours, with total loss of viability after extended incubation over 34 days, consistent with microbial population in the post-stationary phase. Load elimination tests confirmed the effectiveness of the autoclaving process, with no microbial growth during the 48-hour and 36-day incubations.

Therefore, the results of the qualitative tests immediately after the 48-hour incubation were consistent with the three simulated autoclaving conditions, indicating their relevance in routine analysis of GAL-IPEN, CARD-IPEN, and GERADOR IPEN-TEC bioindicators.

Qualitative tests after the decay period proved inadequate due to viability losses on the remaining microorganisms from autoclaving. However, it cannot be ruled out that shorter incubation times and/or different temperatures other than 60 ± 1 °C (32.5 ± 2.5 °C, 22.5 ± 2.5 °C or 5.0 ± 3.0 °C, for example) may result in the maintenance of microbial viability. Additional work may be carried out to test different incubation conditions. The fact that the estimated residual activity is below the release limit for the radionuclides Ga-67, Tl-201, and Mo-99 indicates that opening the ampoule at the end of incubation poses a moderate radiological risk, manageable with ordinary radiation protection measures (use of gloves and appropriate attire for controlled areas, proper disposal of analytical waste).

4. Conclusions

The qualitative membrane filtration test for the detection of *G. stearothernophilus* in the Sterikon® plus bioindicator ampoules is suitable for evaluating vials that have been autoclaved and incubated at 60 ± 1 °C for 48 hours. This test is applicable to vials with contents showing a color range from light red to reddish-purple (effective sterilization) and from yellowish to yellowish-orange (ineffective sterilization). We recommend standard radiation protection measures while handling the ampoules, as they typically contain traces of radionuclides with activity below the release limits recommended by CNEN NN 8.01 standard.

References

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