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Effectiveness Test of Disinfectants to Eliminate Biofilm in Radiopharmacy

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1. Introduction

Biofilms consist of sessile microbial cell communities embedded in extracellular polymeric matrices adhered to substrates, interfaces and many kind of surfaces [1]. The presence of biofilms is critical in medical environment and pharmaceutical production areas, given the possibility of biofilm detaching from adherent surfaces and to be transported to distant points, spreading microbial contamination.

Industrial radiopharmacy faces the same difficulties as the conventional pharmaceutical industry to control biofilms in production areas, mainly where systems containing water are present. Specific literature is almost non-existent, encouraging studies about the theme. Stepanović [2] suggested a criterion to classify bacterial species into non-producers, weak, moderator, and strong producers biofilm formation potential. Due the resistance to mechanical or chemical removal with antimicrobials, the use of effectiveness disinfectant to eliminate or control biofilms is essential [3].

The objective of this work was to classify 23 microorganism biofilms from a non-radioactive radiopharmaceutical production area and to propose a test to evaluate the effectiveness of some disinfectants to eradicate strong microbial contaminant.

2. Methodology

2.1 Preparation of isolated microorganism for biofilm formation and classification [2].

Each isolate of 23 microorganisms in TSA had a suspension of 10^8 cells mL^{-1} prepared in 0.9% NaCl (24 h, 36 ± 1 °C). 20 µL of each suspension were distributed in nine-well replicates of a 96-well microplate, plus 180 µL of trypticase soy broth (TSB; Merck-Millipore). As negative control (NC), 20 µL of 0.9% NaCl were added to nine-well in place of microorganism sample. The microplate was incubated in a humid chamber at 36 ± 1 °C for 24 h, and washed three times with pH 7.2 phosphate saline buffer.

The biofilm was fixed by adding 150 µL of methanol/well during 20 minutes, and air drying for 18 h, followed by 150 µL/well of crystal violet dye for Gram (Merck), washing the microplate with purified water, air drying and adding of 150 µL/well of 95% ethanol (w/w). After 20 minute resting time, the absorbance was measured with Thermo Multiscan EX spectrophotometer at 540 nm wavelength. The final absorbance value for the species (A_F) was expressed as A_C subtracted from the average absorbance value in the test wells (A_T). Negative values of A_F are taken as zero, while any positive value indicates biofilm production. The mean absorbance value in the negative control

wells (A_{CN}) was used to calculate the threshold value (A_C), defined as three standard deviations of A_{CN} above A_{CN}. The classification of a microorganism as biofilm producer was based in the absorbance, as follows: $A_T \leq A_C$, non-producer; $A_C < A_T \leq 2.A_C$, weak producer; $2.A_C < A_T \leq 4.A_C$, moderate producer; $4.A_C < A_T$, strong producer.

2.2 Evaluation of antibiofilm activity of disinfectant on *Micrococcus lylae*

In 15 mL conical tubes containing 1 g of glass beads previously washed with acid, a $1.5x10^8$ cells.mL^{-1} suspension of *Micrococcus lylae* prepared in saline (24 h, 32.5 ± 2.5 °C) and 5 mL of TSB were added [4]. The tubes (in triplicates) for biofilm formation on the surface of the beads for the disinfectant test and positive control were incubated at 37.5 ± 2.5 °C for 24 h. After three washes with saline, without agitation of the beads, those were transferred to a new conical tube, to which 2 mL of disinfectant (test) or saline (positive control) were added. After resting for 10 minutes at room temperature, the beads were washed again, transferred to a conical tube with 5 mL of saline and vortexed at maximum speed (Vortex Genius3 - IKA) for 5 minutes. The concentration of microorganisms in the supernatant was determined by serial dilution in saline (1:10, 1:100, and 1:1,000) and seeding of 100 µL of the dilutions on TSA plates, incubated at 32.5 ± 2.5 °C for 48hours.

Isopropyl alcohol 70% (w/w) Oxivir Five (hydrogen peroxide 4.25%, diluted in purified water at 1:16, 1:64 and 1:128) and Voxilon AN (peracetic acid in 1,000 ppm and 2,000 ppm concentrations) was evaluated as antibiofilm activity of bacteriophages using adapted technique proposed by Gomaa and collaborators [5].

3. Results and Discussion

Table I shows the classification of 23 isolates of microorganism as producer of biofilm based in Stepanović procedure.

Table I: Classification of capacity of microorganism in formation of biofilm.

Microorganism			
Non-producers	Weak producers	Moderate producers	Strong producers
B. atrophaeus *B. faciens* *B. subtilis* *E. coli* *K. schizophila* *M. luteus* *P. luteola* *S. capitis* *S. aureus* (environmental isolate) *S. enterica* subsp. *enterica* ser. *typhimurium* *S. salivarius ssp salivarius*	*B. cereus* *B. megaterium* *B. mycoides* *C. afermentans* *C. parapsilosis* *P. alvei* *S. cohnii ssp urealyticu* *S. hominis* *S. paucimobilis*	*B. choshinensis* *C. albicans* *K. varians* *P.aeruginosa (P.paeruginosa)* *P. macerans*	*B. circulans* *M. lylae* *S. aureus* (NCTC 10788 reference strain *S. saprophyticus*

No biofilm producers was majored (37.9% of species), while strong producers constituted the minority group (13.8%) and *Micrococcus lylae* was characterized as the strongest biofilm producer.

Production behavior varies notably within the same genus, as in the case of *Micrococcus sp.*, and even between individuals of the same species. Four strong biofilm-producing species are known to be associated with bacterial communities present in a variety of environments, from dental

equipment [7] and aluminum alloy corrosion points [9] to food and infectious foci in humans [8]. The bacterium *S. aureus* stands out, whose environmental isolate was shown to not produce biofilm, while the commercial strain was classified as a strong producer. This variation can be attributed to the adaptation of microorganisms to environments with varied nutrient distribution and climatic conditions, implying infraspecific genetic modifications and different biofilm-producing phenotypes. Genetic modulation of biofilm structure may be a fundamental step in bacterial pathogenicity, as demonstrated by Lawal and colleagues for *S. saprophyticus* [8].

The great adaptation capacity of biofilms to adverse environments highlights the need to evaluate the effectiveness of disinfectants against isolates from the production area, possibly adapted to it.

Table II shows the results of antibiofilm activity of disinfectant of *Micrococcus lylae* isolate, a strong biofilm producer.

TABLE II – Antibiofilm activity of disinfectants against *Micrococcus lylae* isolate

Disinfectant	Replicate	$\log_{10}$ [T]	$\log_{10}$ [CP]	$\log_{10}$ [CP] - $\log_{10}$ [T]
Isopropyl alcohol 70% (w/w)	1	2.40	2.75	0.35
	2	2.54		0.21
	3	2.40		0.35
Oxivir Five 1:16 dilution	1	< 1.70	2.75	> 1.05
	2	< 1.70		> 1.05
	3	< 1.70		> 1.05
Oxivir Five 1:64 dilution	1	< 1.70	3.12	> 1.42
	2	< 1.70		> 1.42
	3	< 1.70		> 1.42
Oxivir Five 1:128 dilution	1	< 1.70	3.12	> 1.42
	2	< 1.70		> 1.42
	3	< 1.70		> 1.42
Voxilon AN 2,000 ppm	1	2.65	2.75	0.10
	2	2.30		0.45
	3	2.40		0.35

Subtitle: [T] and [CP], bacterial concentrations ($CFU.mL^{-1}$) of biofilms dispersed in saline, for test and positive control (average of replicates), respectively.

The antibiofilm activity of a disinfectant is present when the reduction of the concentration of microorganisms in the test is greater than or equal to $2.\log_{10}$ of the positive control - criterion adopted by Harrison and collaborators in a study on the antibiofilm activity of *Pseudomonas aeruginosa (P. paraeruginosa)* [6].

In TABLE II, 70% isopropyl alcohol and Voxilon AN (2,000 ppm) disinfectants did not show antibiofilm activity against *M. lylae*, while the results for dilutions of Oxivir Five were inconclusive. The resistance of biofilms to high and medium level disinfectants is notable, as in the case of *S.aureus* against alcohols, hydrogen peroxide and peracetic acid in the presence of organic matter [10] or the biofilm of *Pseudomonas aeruginosa (P. paraeruginosa)* briefly exposed to peracetic acid .

Preliminary results demonstrate that the set of disinfectants used in a given production areas must be tested against isolates from the area itself, under standardized experimental conditions. Cases of recurrent contamination of clean rooms with the same species of microorganism – possibly associated with biofilm resistance – can be treated by selecting the most effective disinfectant against the species. The contact time between disinfectant and biofilm can be adjusted in the proposed test, in order to optimize the reduction in the population of microorganisms. The time

thus optimized can be adopted as a parameter in the sanitization of clean rooms. The antibiofilm activity test should also cover weak producers, since pathogenic species may be present in that group (for example, *B. cereus* in this work).

4. Conclusions

Biofilm non-producer microorganisms were predominant into the spectrum of evaluated isolates. Among the strong producers, the bacterium *Micrococcus lylae* presented a biofilm resistant to the 70% isopropyl alcohol and Voxilon AN 2,000 ppm disinfectants. The methodology presented in this work can be extended to isolates from hot cells and glove boxes, in order to characterize the typical microbial flora of radioactive radiopharmaceutical production and to select the most appropriate disinfectants for each production area.

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