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Solid phase cytometry applied to sterility tests for injecting 0.9% sodium chloride

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Sterility tests described in official compendia are carried out by membrane filtration or by direct inoculation into suitable culture media. About 14 days are needed to provide results and release products for sale, so speed is of the essence in rapid microbiological methods. Solid phase cytometry is a fast innovative method for testing the sterility of injectable medications. It is based on the detection of viable cells by using reagent viability markers which permeate the cell membrane, and are cleaved by non-specific sterases to form fluorochrome, which is detected by a Chem Scan RDI®. This study set out to evaluate this technology when applied to the sterility test in a 0.9% sodium chloride injection solution, using Chem Scan RDI® equipment. Microorganisms recommended by the official compendia *Clostridium sporogenes* NCTC12935 (ATCC 11437), *Pseudomonas aeruginosa* NCTC12924 (ATCC 9027), *Staphylococcus aureus* NCTC10788 (ATCC 6538), *Bacillus subtilis* NCTC10400(ATCC 6633), *Aspergillus brasiliensis* NCPF2275 (ATCC16404) and *Candida albicans* NCPF3179(ATCC 10231), and two "in house" microorganisms, *Micrococcus luteus* and *Staphylococcus epidermidis,* obtained from monitoring the pre-sterilization bioburden, were evaluated in order to validate the proposed method. When the solid phase cytometry method was compared to the traditional membrane filtration sterility test for all the microorganisms tested, it was found to be significantly faster in that it reduced analysis time from 14 days to approximately 3 h.

Key words: Solid phase cytometry, sterility test by membrane filtration, validation.

INTRODUCTION

Microbiological methods can be used for many purposes in the pharmaceutical industry, and among their possible uses are the following: determining the microbial load in non-sterile products and raw materials; the environmental

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monitoring of air, water, surface and people; sterility tests on products for parenteral and ophthalmic use; antimicrobial effectiveness tests and identification of pathogens in raw materials and finished products (Riley, 2004; Pinto et al., 2010).

Classical microbiological methods are based on microbial growth, and depend on the visual observation of the presence of microorganisms through the turbidity of the culture media or colony formation, followed by microscopic observation. Although they are quite effective, they have certain disadvantages such as the delay in presenting results, which is influenced by the growth conditions of the culture media, low repeatability and reproducibility and the inability to detect viable but not culturable microorganisms (VBNC) (Lemarchand et al., 2001; Silveira, 2006; Evangelista, 2008; Moldenhauer, 2008; Vanhee et al., 2008; Pinto et al., 2010).

Of the classical microbiological methods, the traditional sterility test by membrane filtration (MF) has been used for decades to ensure product sterility. However, its limitations, particularly in relation to the growth properties of the medium used in the test, have been questioned and has its incubation time of 14 days, which is considered relatively high (Brasil, 2010; Pinto et al., 2010).

Due to growing pressure for immediate results, rapid microbiological tests began to emerge from the 1970s onwards. Their main objectives were to improve efficiency, simplify the work involved, make the tests less time consuming, enhance their analytical capacity, increase their reliability and intensify result accuracy. From the pharmaceutical point of view, rapid or alternative microbiological methods can be used for the quality control of products, the environmental monitoring of clean rooms, microbial counts, efficiency testing of preservatives, sterility tests, water analysis, microbial identification and characterization and the microbiological testing of in-process control. In short, they are used to quantitatively or qualitatively assess microbial contamination or to get a quick confirmation of the absence of microorganisms (Shintani et al., 2011; Parenteral Drug Association, 2013).

Rapid methods involve microorganism detection technologies, and can be based on the monitoring of growth, cell viability, cellular components or nucleic acids (Pinto et al., 2010; Duguid et al., 2011; Parenteral Drug Association, 2013). Solid phase cytometry (SPC) is a method based on cell viability, characterized by its rapid analysis and high sensitivity for microbiological control in the pharmaceutical industry. It is performed by direct fluorescence labeling of viable microorganisms coupled to a laser and an ultrasensitive scanning counting system, which can detect a single cell, and thereby eliminate the need for cell growth. Fluorescence is obtained through the cleavage of carboxyfluorescein diacetate by intracellular esterase enzymes, to yield fluorescent carboxyfluorescein in metabolically active cells (Parthuisot et al., 2000; Smith et al., 2010). After labeling the membrane filters, fluorescence is detected by the ChemScanRDI® equipment (488 nm) and signals are emitted and processed using TVB Bio software (Chemunex) that allows for differentiation between the target cells and background fluorescence. Each “spot” is visually inspected and detected as a target cell using an epifluorescence microscope (Nikon Eclipse 50i, Tokyo, Japan) (Smith et al., 2010; Dupont and Augustin, 2011).

The primary advantage of SPC as compared to the MF traditional method for sterility testing is its ability to provide faster results, which is well below the time required for the traditional methods described in the official compendia (Parthuisot et al., 2000; Brasil, 2010; Méheust et al., 2013; United States Pharmacopeia, 2013). Assays that allow for the detection of metabolically active cells are attractive because they can detect not only viable microorganisms, but also VBNC (Diaper et al., 1992; Jacobsen et al., 1997; Parthuisot et al., 2000; Cools et al., 2005; Dupont and Augustin, 2011).

The purpose of the validation of the rapid SPC method was to prove the equivalence between it, and the traditional membrane filtration method for 0.9% sodium chloride injection solution. The use of SPC is justified by the need for a method which is efficient, reliable, accurate, and which enhances analytical capacity, thereby making the work easier and not just reducing the time and costs involved (Silveira, 2006).

MATERIALS AND METHODS

Samples and sterility test methodology

A total of 10 sterile samples were used for all tests. For specificity test, 10 non-sterile samples from 3 batches of the 0.9% sodium chloride injection solution were also included used for the solid phase cytometry methods.

Microorganisms and preparation of conditions

The standard microorganisms used were *Aspergillus brasiliensis* NCPF 2275 (ATCC 16404), *Bacillus subtilis* NCTC 10400 (ATCC 6633), *Candida albicans* NCPF 3179 (ATCC 10231), *Clostridium sporogenes* NCTC 12935 (ATCC 11437), *Pseudomonas aeruginosa* NCTC 12924 (ATCC 9027), *Staphylococcus aureus* NCTC 10788 (ATCC 6538), obtained from quantitative lyophilized strains of the BIOBALL® brand in a concentration of 550 CFU (BIOBALL® MultiShot 550, Biomerieux, France) and two “in house” microorganisms, *Micrococcus luteus* and *Staphylococcus epidermidis,* obtained from the pre-sterilization bioburden, identified by biochemical tests (BBL CRYSTAL®, BD, USA). “Stock suspensions” of each standard microorganism were prepared and diluted in 0.1% peptone water yielding suspensions with concentrations of 0.5 CFU/ml, 2.0 CFU/ml, 5.0 CFU/ml and 50 CFU/ml, where the 50 CFU/ml suspensions were quantified by plating in depth for all listed microorganisms, using Soybean-

Casein Agar (Difco) for bacteria and Sabouraud-Dextrose Agar (Difco) for fungi, incubated at 32.5±2.5 and 22.5±2.5°C, respectively.

The “in house” microorganism suspensions were prepared from isolated colonies, diluted in 0.85% sterile sodium chloride (w/v), followed by serial dilutions of 10^{-1} to 10^{-6}, which were quantified by plate method using Soybean-Casein Agar (Difco). A total of 10 replicates were made for each microorganism.

Solid phase cytometry method

The “pool” of 10 samples of 0.9% sodium chloride injection solution (250 ml in total) was filtered through black polyester membranes with a diameter of 25 mm and a porosity of 0.4 µm (ChemFilter CB04). After filtration, the membranes were separately inoculated with 1 ml of each concentration of the evaluated microorganisms between the first and second rinsing with 0.1% peptone water, followed by the addition of 1 ml of counter staining solution (CSM Reagent:FSC Reagent) to minimize background fluorescence. The membrane was transferred to a disk (support pad), saturated with 550 uL of medium A16 ChemSol activation, incubated at 30°C±2°C for 2 h, and transferred to a disk (pad holder) containing 550 uL of the marking solution (ChemSol B16 + ChemChrome V6) and again incubated at 30°C±2°C for 45 min. After labeling the microorganisms, the membrane filter was transferred to a holder, placed under a disk (support pad), containing 100 µL of ChemSol B16 solution, and scanned by ChemScan RDI® (AES Chemunex, France) (488 nm).

The detection of viable microorganisms was confirmed through visual inspection, using an epifluorescence microscope (ECLIPSE CI-S/940,293-NIKON®). The above-mentioned materials and reagents were obtained from AES Chemunex-Biomerrieux, France.

Sterility testing by membrane filtration

The tests were performed using the same microorganism suspensions used in the SPC method. After filtration of the 10-sample “pool” of 0.9% sodium chloride injection solution (1000 ml in total), using a membrane filter with a diameter of 50 mm and 0.45 µm pore polyester membrane (Millipore/Merck – Germany), 1 ml of each concentration of evaluated microorganisms was inoculated into separate membranes between the first and second rinsing with sterile 0.1% peptone water.

The membrane was transferred to a tube containing 100 ml of the specific culture medium for each type of microorganism evaluated and incubated for up to 14 days at 32.5°C±2.5°C (Fluid Thioglycollate - Difco) and 22.5°C±2.5°C (Soybean-Casein Broth - Difco), for bacteria and fungi, respectively. Readings were taken daily, until the media acquired turbidity.

Validation

Specificity

Specificity was carried out in three phases for the SPC method only. First, an evaluation of each viable and non-viable microorganism (using an autoclaving process at 121°C for 15 min) was done in the ChemScan RDI® (AES Chemunex, France) (488nm), in the absence of the sample. A total of 7 replicates were made for each microorganism at a concentration of 50 CFU/ml. In the second phase, the microorganisms were evaluated in the presence of a sterile sample at concentrations of 0.5, 2.0, 5.0 and 50 CFU/mL. And in the third phase, the non-sterile samples were evaluated without the presence of microorganisms. In these tests, 3 batches were used for 0.9% sodium chloride injection solution with 7 replicates for each microorganism.

Detection limit

The SPC and MF methods of analysis were performed in parallel using the same suspensions for each of the microorganisms in concentrations of 0.5, 2.0, 5.0 and 50 CFU/mL, respectively with 3 batches of 0.9% sodium chloride injectionsolution and 7 replicates.

Robustness

The robustness of the SPC method was tested with 7 replicates of *A. brasiliensis* NCPF 2275 (ATCC 16404), *B. subtilis* NCTC 10400 (ATCC 6633), *S. aureus* NCTC 10788 (ATCC 6538) and *P. aeruginosa* NCTC 12924 (ATCC 9027), at a concentration of 2 CFU/mL, and with 1 batch of 0.9% sodium chloride injection solution, where changes in membrane reading time (5, 7 and 20 min) in the ChemScan RDI® and variations in volumes of pre-labelling buffer solution ChemSol A16 - AES Chemunex (530, 550 and 570 µL) were evaluated.

Statistical analysis

The model of logistic regression and chi-square tests of homogeneity were used to evaluate the sensitivity and equivalence between the methods by analyzing and comparing the number of positive growth cultures detected by the MF and SPC methods for each concentration of eight microorganisms. All statistical analyses were performed using the portal action software (Action, 2014).

RESULTS

Identification of the “in house” microorganism

Identification of the “in house” microorganismsby biochemical evidence contained in the BBL CRYSTAL® GP kit presented a 99.3% degree of accuracy for *S. epidermidis* and 99.9% for *M. luteus.*

Evaluation of the quantity of microorganisms after preparation of the stock suspension

From the average of the results obtained in the plating of the suspension stocks of lyophilized and “in house” microorganisms performed with 10 replicates of each microorganism in the concentration of 50 CFU/ml, using soybean-casein agar (Difco) for mesophilic bacteria, blood agar (Difco) in anaerobic jars plus Anaerocult A (Merck-Millipore) for anaerobic and sabouraud-dextrose agar (Difco) for fungi, the count found was within the parameters recommended by the official compendium that allows for a standard deviation of <15% at concentrations of between 30 and 300 CFU/plate (Table 1).

Table 1. Plating the stock suspensions in the concentration of 50 UFC/ml.

Microorganisms	Medium value of the plate count	Standard deviation (%)
Aspergillus brasiliensis	49.5	1.91
Bacillus subtilis	50.6	1.62
Candida albicans	49	1.61
Clostridium sporogenes	47.8	1.62
Pseudomonas aeruginosa	50.6	1.64
Staphylococcus aureus	50.2	1.62
Micrococcus luteus	50.3	5.29
Staphylococcus epidermidis	46.9	4.01

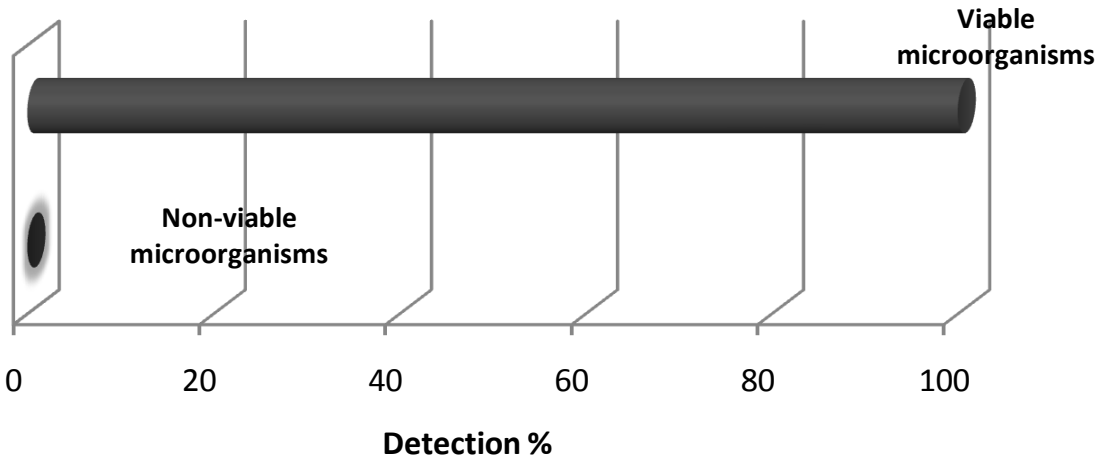


Figure 1. Viable and non-viable microorganism detection by SPC.

Specificity

As shown in Figure 1, it can be seen from the analyses that the ChemScan RDI® can differentiate between viable and non-viable microorganisms, since there was 100% detection of all viable cells, while the non-viable cells (inactivated by the autoclaving process at 121°C for 15 min) were not detected. From the results shown in Figure 2, it was certified that the 0.9% sodium chloride injection solution did not interfere with the recovery of the microorganisms evaluated at concentrations of 0.5 CFU/ml, 2.0 UFC/ml, 5 CFU/ml and 50 CFU/ml. At the lowest concentration of 0.5 CFU/ml, the lowest recovery was 42.85% for *S. epidermidis*, and the highest 76.19% for *C. sporogenes*. For the other concentrations, the detection was 95.23% for all tested microorganisms.

After analyzing 0.9% non-sterile sodium chloride solution, it was shown that the SPC method is capable of recovering microorganisms present in the sample prior to sterilization, and of ensuring that, using an epifluorescence microscope (ECLIPSE CI S / 940 293 - NIKON), it is possible to distinguish the self-fluorescent particles in the sample of viable microorganism cells (Figure 3).

Detection limit

To determine the detection limit, four concentrations (0.5, 2.0, 5.0 and 50 CFU/mL), two detection methods (MF and SPC), 8 microorganisms, 3 batches of products and 7 repetitions in each combination were used, which resulted in 168 tests for each concentration, and a total of 1.344 tests. At concentrations of 0.5, 2.0 and 5.0 CFU/ml, percentage detection rates of 61.3, 79.2 and 98.8%, respectively, of viable cells were found for the SPC method, whereas with the MF method and the same concentrations, percentages rates of 42.2, 58.9 and 84.5%, respectively, were obtained. With the concentration of 50 CFU/ml, both methods reached a 100% detection percentage rate (Table 2). It was seen that at a low concentration of microorganisms, the SPC method provided better detection than the MF method, whereas for a higher microbial concentration, detection

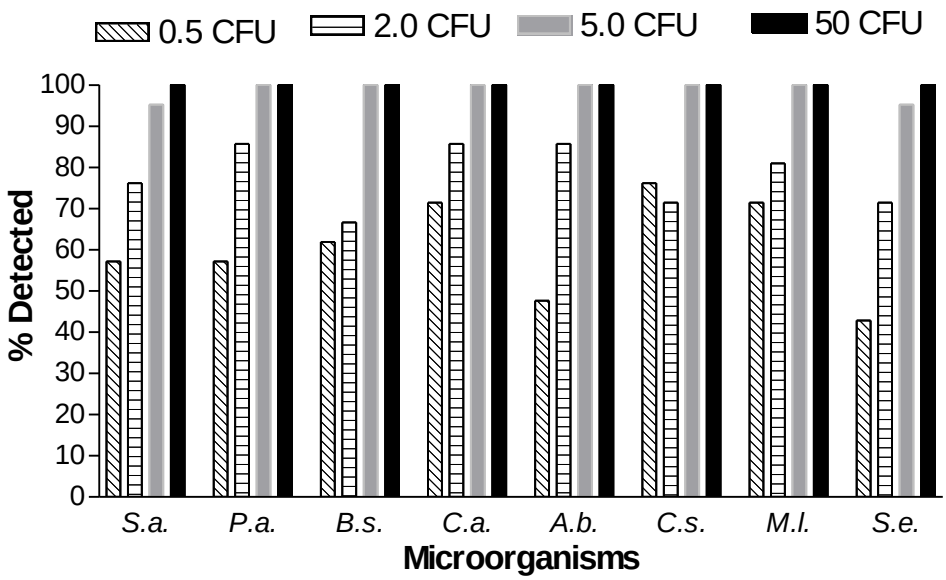
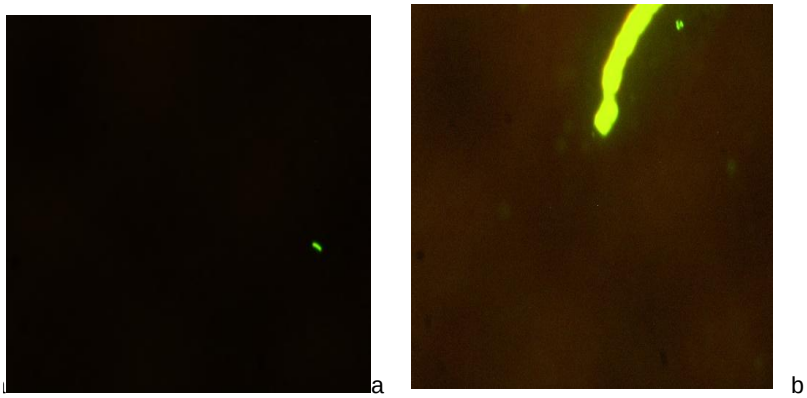


Figure 2. Detection of microorganisms by the ChemScan RDI in the presence of 0.9% Sodium Chloride injection solution. *Sa* (*Staphylococcus aureus*); *P.a*. (*Pseudomonas aeruginosa*); *S.e*.(*Staphylococcus epidermides*); *M.l*. (*Micrococcus luteus*); *C.s*. (*Clostridium sporogenes*); *B.s*. (*Bacillus subtilis*); *C.a*.(*Candida albicans*); *A.b*. (*Aspergillus brasiliensis*).



a b

Figure 3. Epifluorescence-microscopy (ECLIPSE CI-S/940,293-NIKON®)image of *B. subtilis* (a) and autofluorescent particle (b)

rates for both methods were similar.

As described in Table 3, it can be seen that the detection limit for the SPC method was 3.87 and 7.79 CFU for the MF method with a 95% detection probability, as calculated by the logistic regression model. The results of this study, undertaken to determine the detection limit, were used to apply the homogeneity chisquare test, without taking the results of each concentration of microorganisms (Table 4) into account separately,as the behavior of the results differs according to the concentration of the microorganisms in the test, that is, at low concentrations, the SPC method gives better results, whereas at higher concentrations the methods are similar, the chi-square test had difficulty in detecting differences between the methods. However, this test was used to show that, irrespective of this restriction, differences between the SPC and MF methods were also detected (Table 5).

Robustness

By analyzing the time variation of the membrane readings with a concentration of 2 CFU/ml, it was seen that after 5

Table 2. Microbial detection results with concentrations of 0.5, 2.0, 5.0 and 50 CFU/ml with all eight microorganisms by MF and SPC methods.

Method	Total analysis	Concentrations			
		0.5	2.0	5.0	50.0
SPC presence	672	103	133	166	168
Percentage rates		61.3	79.2	98.8	100
MF presence	672	71	99	142	168
Percentage rates		42.2	58.9	84.5	100

Table 3. Detection limits for the SPC and MF methods obtained by statistical analysis using logistic regression.

Methods	Concentration	Probalility of detection	Lower limit	Upper limit	Standard deviation
SPC	0.5	0.59	0.52	0.66	0.04
	1.0	0.68	0.63	0.73	0.03
	2.5	0.87	0.83	0.91	0.02
	2.9	0.90	0.86	0.94	0.02
	3.0	0.91	0.87	0.94	0.02
	3.87*	0.95*	0.92*	0.98*	0.01*
	6.5	0.99	0.99	1.000	0.00
MF	0.5	0.42	0.36	0.49	0.03
	5.0	0.85	0.79	0.90	0.03
	6.0	0.90	0.85	0.94	0.02
	7.0	0.93	0.89	0.97	0.02
	7.79*	0.95*	0.92*	0.98*	0.02*
	9.0	0.97	0.95	0.99	0.01
	13.0	0.99	0.99	1.0	0.003

*Numbers show detection limits found for the SPC and MF techniques with detection probability of 95%.

Table 4. Analyses for determining the detection limit with eight micro organisms in 3 batches of 0.9% sodium chloride injection solution, with the SPC and MF methods.

Methods	Positive	Sterile	Total	Sterile (%)	Positive (%)
SPC	570	102	672	15	85
MF	480	192	672	29	71
Total	1050	294	1344	-	-

and 7 min there was a detection of more than 70% for all the microorganisms evaluated, except for *B. subtilis,* where the detection reached 57.14% after 7 min. After 20 min, the detection was less than the 42.86% for all the microorganisms, and *B. subitilis* was not detected at all (Figure 4). For the volume change of the pre-labelling solution (A16 ChemSol), detection was more than 70% for all microorganisms, with volumes of 530 and 570 µl similar to the detection of the volume of 550 µl, which is recommended by the method (Figure 5). The results of the robustness tests were analyzed by the chi-square test and significant differences could be seen between the reading times of the membrane in 5 to 20 and 7 to 20 min (Table 6). This did not occur with the variation of volume of the pre-labelling solution (ChemSol A16 - AES Chemunex, France), where at a significance level of 5%, there was no significant difference between the results (p = 0.783896517).

Table 5. Chi-square test of homogeneity applied to the results of the detection limit of the SPC and MF methods at concentrations of 0.5, 2.0, 5.0 and 50 CFU/ml with the eight microorganisms tested.

Statistics X^2	*p*-value
34.49	4.29 E-09

Time required for detecting the growth of microorganisms

As described in Table 7, the time required by the SPC method was about 3 h, irrespectiveof suspension concentration, whereas for the MF method the shortest time required for the detection of *P. aeruginosa* growth was 24 h at a concentration of 5.0 CFU/ml, and of 216 h at a concentration of 0.5 CFU/ml.

DISCUSSION

In order to ensure the sterility of injectable pharmaceutical products, the sterility test must be completed and, in addition, it is necessary to have control of the environmental monitoring of air, surfaces, people and product pre-sterilization bioburden. Despite the fact that the traditional method is very efficient and has often been used for the release of injectable products, it has the disadvantages mentioned above (Pinto et al., 2010; Vanhee et al., 2009a). Thus, with the need to simplify the work involved, reduce the time consumed, and increase analytical capacity, reliability and accuracy, it is believed that rapid methods can yield results with the same degree of security and speed (Silveira, 2006).

To evaluate the detection of microorganisms, there are different rapid technologies on the market such as methods based on bacterial growth, the direct measurement of cell viability and analysis of cellular components. However, in order to apply them to pharmaceuticals products, one should consider factors such as the principles and expertise inherent in the method, adequate training of professionals and compatibility with the product to be analyzed (Brasil, 2010). If a new method is to be used as proof of the sterility of pharmaceutical products, tests are required to show the non-inferiority between alternative and traditional methods. According to official compendia, the parameters for qualitative tests to be performed during the validation of an alternative microbiological method are specificity, detection limit and robustness (United States Pharmacopeia, 2013).

In this study, the SPC method was selected for conducting the sterility test by presenting the membrane filtration step, which is required for the sterility testing of large volume parenteral solutions in official compendia and must be sensitive enough to detect a single cell. For the specificity parameter, the microorganisms used were those mentioned in the official compendia, including Gram-negative, Gram-positive, aerobic, anaerobic, spore-forming, yeasts and fungi, in addition to microorganisms isolated through pre-sterilization bioburden. As the aim of sterility testing is to determine small microbial loads, it is vital that the microbial suspensions being used are monitored and that they provide a standard deviation of less than 15% at concentrations of between 30 and 300 CFU/plate. With that in mind, this study results (Table 1) are in accordance with the deviation allowed by current legislation and with the results found by Lira (2013), where the microbial load declared in the certificate of analysis issued by the manufacturer of the strains was confirmed.

One important aspect of SPC is the dye used as a viability marker of microorganisms. The dye used in this study, Chem Chrome V6, containing carboxyfluorescein diacetate is a universal marker widely used to assess cell viability due to its permeability to bacterial cell membrane and the capacity of the diacetate group (CA), to be hydrolyzed in fluorescent carboxyfluorescein (CF) by intracellular esterases, showing superior efficiency to other markers of cell viability reagents (Parthuisot et al., 2000; Hoefel et al., 2003).

The results presented in this research, during the performance of specific tests, clearly show that with this marker it was possible to differentiate between viable and non-viable microorganisms, thereby confirming data obtained in a study by Hoefel et al. (2003), which evaluated its efficacy through a comparative study of the above-mentioned dye and Carboxyfluorescein Diacetate Succinimidyl Ester (CFDA/SE) in cultured bacteria, such as *Aeromonas hydorphyla, Bacillus subtilis, Escherichia coli, Pseudomonas aeuruginosa, Staphylococcus epidermidis* and environmental water bacteria. Through analyses in the exponential phase, of the cultures, mixtures of inactive and active cells and environmental bacteria from the water, it was concluded that the CFDA marker was efficient in detecting bacterial activity, while the CFDA/SE, despite having higher intracellular retention due to the presence of the succinimidyl ester group (SE), is strongly linked to intracellular amines, and labels inactive and active cells with the same intensity, and thereby causes unspecific staining results for all cells. One proven significant finding from the specificity assays in this study was the non-interference of the sample tested in the detection of microorganisms (Figure 2), giving no bacteriostatic or fungistatic activity for the product analyzed.

This study can conclude from the results obtained in determining the detection limit (Table 2) that with low concentrations of microorganisms (0.5, 2.0 and 5.0 CFU/mL), SPC presents greater detection capacity than

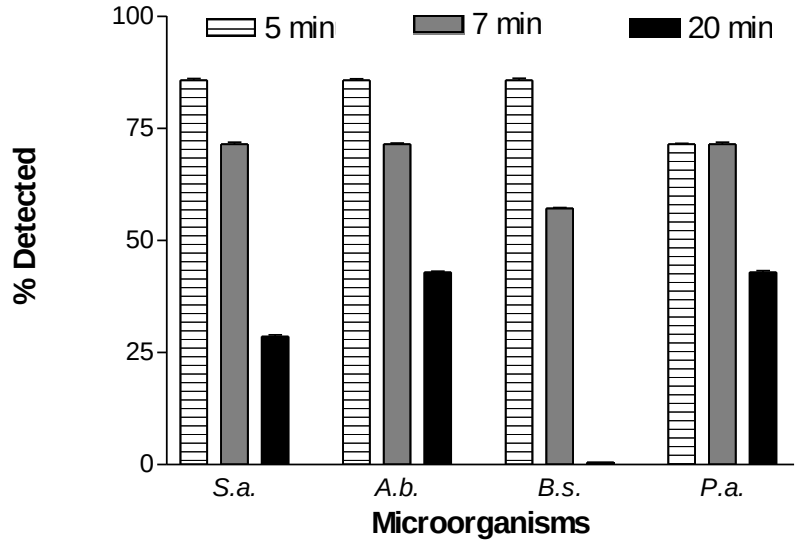


Figure 4. Evaluation of robustness of the membrane with different reading times (5, 7 and 20 min.) for *S. aureus* (*S.a.*), *A.brasiliensis* (*A. b.*), *P. aeruginosa* (*P.a.*) and *B. subtilis* (*B.s.*) at a concentration of 2 CFU/mL.

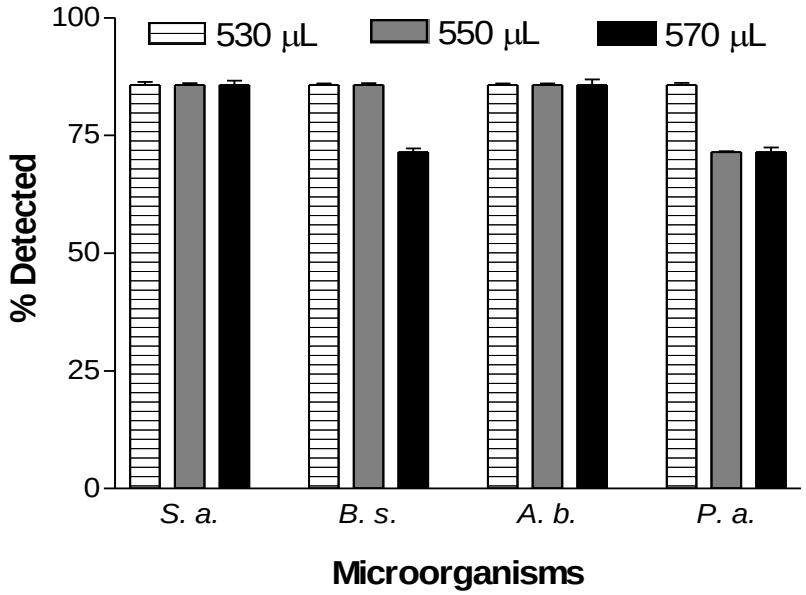


Figure 5. Evaluation of robustness with different volumes of pre-labelling solution (530, 550 and 570 uL) for *S. aureus* (*S.a.*), *A. brasiliensis* (*A.b.*), *P. aeruginosa* (*P.a.*) and *B. subtilis* (*B.s.*) at a concentration of 2 CFU/mL.

Table 6. Chi-square test applied to robustness tests in the range of membrane reading time at a concentration of 2 UFC/ml with four microorganism tests.

Reading time (min)	Total assays	Statistics X^2	*P*-value
5-7	56	1.456910569	0.227422
5-20	56	14.16258065	0.000168
7-20	56	5.815384615	0.015887

Table 7. Time required for detecting viable microorganisms using MF and SPC methods at concentrations of 0.5 and 5.0 CFU/ml.

Micro organism	CFU/ml in sterile peptone water 0.1%	Time required to detect viable cells/growth (h) (Average)	
		Membrane filtration	Solid phase cytometry
S. aureus	0.5	120	3
	5.0	72	3
B. subtilis	0.5	72	3
	5.0	72	3
P. aeruginosa	0.5	216	3
	5.0	24	3
C. sporogenes	0.5	72	3
	5.0	72	3
A. brasiliensis	0.5	96	3
	5.0	96	3
C. albicans	0.5	72	3
	5.0	60	3
M. luteus	0.5	72	3
	5.0	48	3
S. epidermidis	0.5	72	3
	5.0	48	3

*Numbers represent the highest and lowest detection time required by the MF method.

the MF method, proven through statistical analysis. The logistic regression model, with a 95% confidence limit, in which we separately evaluated four microbial concentrations, showed a 3.87 CFU detection limit for SPC and 7.79 CFU for MF (Table 3). In the homogeneity chi-square test, when comparing the two methods with concentrations of 0.5, 2.0, 5.0 and 50 UFC/ml (Table 4), a *p*-value of 4.29E-0.9 (Table 5) was obtained, which showed a significant difference between the methods in terms of detection capabilities. The study concluded that the rapid method has a detection capacity greater than that of the traditional method.

The study results confirm those of Smith et al. (2010), who also evaluated SPC as an alternative method for sterility testing and concluded that it is numerically superior and statistically not less than the compendial sterility test in terms of detection limits for all microorganisms tested. Low detection limits, speed and ability to enumerate all viable microorganisms were also found for the SPC method, when it was compared to the traditional culture method in the quantification of *Aspergillus fumigatus* and in the enumeration of microorganisms in environmental air samples (Vanhee et al., 2008, 2009b).

Microbiological rapid methods, based on the detection of growth by bioluminescence technology Rapid Milliflex Detection System (RMDS), and CO_2 detection (Bact/Alert BioMerieux, France and BACTEC Becton Dickinson, USA, Systems) have also been tested as alternatives to vaccines and other sterility tests for biological products, using the inoculum of various microorganisms at 0.1, 1.0, 10 and 100 CFU. Although the RMDS is a different detection methodology, its results were the same as those obtained in this study, since it was significantly more sensitive and faster in detecting multiple microorganisms at 1.0 and 0.1 CFU than the compendial method (MF) and Direct Inoculation (DI) ($p < 0.05$). The same did not apply to the CO_2 detection method (BACTEC and Bact/Alert), since the compendial method (MF) was more sensitive with an inoculum of 1 CFU ($p < 0.01$), and with an inoculum of 0.1 UFC the MF, DI, BACTEC and BacT/Alert methods showed similar sensitivity (Parveen et al., 2011).

The results obtained in the membrane time variations in the robustness assay have shown that can define a limit of up to 7 min for the membrane reading, and variations in volume (±20 uL) of the pre-labelling solution (ChemSol A16 - AES Chemunex, France), without changing the results of the detection of microorganisms by the SPC method. These small variations in their usual parameters are crucial for the validation step of a microbiological alternative method, so that limits can be set when the method is routinely used (Parenteral Drug Association, 2013).

According to international guidelines, if an equivalent method is to be considered, it must provide a guarantee of safety. In addition, proven purity and potency must be equal to or greater than the guarantee given by the approved traditional method, since the planning of the experiment is fundamental if safe and accurate results are to be obtained. Through the results, this study was able to show that the SPC method was specific, that it managed to detect all viable microorganisms tested in the presence of the sample, and differentiate them from non-viable ones. It presented a detection limit below that of the MF method and proved to be much faster as it obtained a result in only 3 h, as compared to 14 days in the compendia MF method. When tested, it was shown to be robust against small variations in membrane reading time and volume of the pre-labelling solution (ChemSol A16 - AES Chemunex, France). This allowed this study to conclude that the alternative microbiological method of Solid Phase Cytometry is equivalent to the traditional method of membrane filtration for sterility testing for 0.9% sodium chloride injection solution.

Conclusions

It was therefore concluded that when the SPC and the traditional MF methods were compared in terms of the time required to detect viable microorganisms, the SPC method detects them significantly faster (3 h) than the traditional membrane filtration method (14 days), thereby demonstrating the equivalence of these methods.

Conflicts of interest

The authors have not declared any conflict of interest.

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Abbreviations

CFU, Colony-forming unit; **SPC,** solid phase cytometry; **MF,** membrane filtration; **VBNC,** viable but not culturable.

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