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Evaluation of fluorescence detection technique as an alternative for heterotrophic bacteria count in hemodialysis water

Avaliação da técnica de detecção por fluorescência como alternativa para contagem de bactérias heterotróficas em água para hemodiálise

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ABSTRACT

Introduction: The microbiological quality of hemodialysis treated water is directly related to the occurrence of infections and pyrogenic reactions in patients. **Objective:** Determine the minimum incubation time and evaluate the alternative microbiological method performance for heterotrophic bacteria count in hemodialysis water through the fluorescence microbial detection technique. **Method:** The analyses were conducted by concentration levels of 2,5 x 10^{-1} to 1,0 x 10^{2} CFU/plate for *Pseudomonas aeruginosa*, *Burkholderia cepacia*, *Escherichia coli* and *Staphylococcus aureus*. The tests were performed simultaneously by the alternative and the traditional methods, using culture medium R2A and incubation temperature of 24.0°C ± 4.0°C. The incubation times were 40 h and 120 h, respectively. Fourteen hemodialysis water samples were analyzed to assess the equivalence between the methods evaluated. **Results:** The results demonstrated that the alternative method allows quantification of heterotrophic bacteria after 40 h of incubation, with accuracy, precision, specificity and linearity for the range of 5 to 100 CFU/plate. The detection limit of the alternative method is 1 CFU/plate. **Conclusions:** It was possible to conclude that the alternative method has equivalent results to the traditional method, since the confidence interval of the alternative method was entirely within the equivalence range. Therefore, the microbial detection technique by fluorescence showed a viable option for the implementation of a rapid microbiological method for the heterotrophic bacteria count in samples of treated water for hemodialysis.

KEYWORDS: Hemodialysis; Treated Water; Heterotrophic Bacteria; Fluorescence

RESUMO

Introdução: A qualidade microbiológica da água tratada para hemodiálise está diretamente relacionada à ocorrência de infecções e de reações pirogênicas nos pacientes. **Objetivo:** Determinar o tempo de incubação mínimo e avaliar o desempenho do método microbiológico alternativo para a contagem de bactérias heterotróficas em água de hemodiálise por meio da técnica de detecção microbiana por fluorescência. **Método:** As análises foram conduzidas com níveis de concentração entre 2,5 x 10^{-1} e 1,0 x 10^{2} UFC/placa para *Pseudomonas aeruginosa*, *Burkholderia cepacia*, *Escherichia coli* e *Staphylococcus aureus*. Os testes foram realizados simultaneamente pelos métodos alternativo e tradicional, utilizando o meio de cultura R2A e temperatura de incubação de 24,0°C ± 4,0°C. Os tempos de incubação empregados foram os de 40 h e 120 h, respectivamente. Quatorze amostras de água para hemodiálise foram analisadas para avaliação da equivalência entre os métodos avaliados. **Resultados:** Os resultados demonstraram que o método alternativo permite a quantificação de bactérias heterotróficas após 40 h de incubação, com precisão, exatidão, especificidade e linearidade para a faixa de 5 a 100 UFC/placa. O limite de detecção do método alternativo é 1 UFC/placa. **Conclusões:** O método alternativo possui resultados equivalentes ao método tradicional, uma vez que o intervalo de confiança do método

alternativo obtido esteve compreendido inteiramente dentro da faixa de equivalência. Portanto, a técnica de detecção microbiana por fluorescência mostrou ser uma opção viável para a implementação de um método microbiológico rápido para a contagem de bactérias heterotróficas em amostras de água tratada para hemodiálise.

PALAVRAS-CHAVE: Hemodiálise; Água Tratada; Bactérias Heterotróficas; Fluorescência

INTRODUCTION

The microbiological quality of water treated for hemodialysis is directly related to the occurrence of infections and pyrogenic reactions in patients. Changes in the integrity of dialyzer membranes and the inadequate maintenance of the water treatment and distribution system stand out as the most prevalent causes of contamination, mainly by Gram negative bacteria – potential biofilm-forming bacteria – which, once established, facilitate the microbial persistence and act as a permanent source of bacteria and endotoxins, thus increasing the risk to which patients are exposed[1,2,3].

Because of the criticality of the quality of water for hemodialysis, Brazil's National Health Surveillance Agency established, through Resolution of the Collegiate Board n. 11, of March 13, 2014, the minimum standard of microbiological quality, which does not accept heterotrophic bacteria counts greater than 100 colony-forming units (CFU) per milliliter (mL)[4].

Most microbial contaminants in water systems are mainly in the form of surface biofilms, with only a small percentage of the microbiome suspended in water in planktonic form[5].

Although the best option is related to the direct monitoring of biofilm growth on surfaces, current technologies for surface assessment in water systems make this task unfeasible in dialysis clinics. Therefore, an indirect approach must be used: an assessment for detection and enumeration of planktonic microorganisms released from biofilm via collection of samples from the water system. By enumerating the microorganisms found in these samples, the overall status of control over the biofilm growth in the water treatment system can be determined[5].

Failure to meet the microbiological quality criteria for hemodialysis water determined by the legislation[4] may result in the occurrence of pyrogenic reactions caused by endotoxins, as well as infections, which are the main cause of morbidity and mortality in hemodialysis patients[6].

The legislation establishes that the dialysis service must monitor the microbiological quality of the water resulting from the water treatment and distribution system for hemodialysis every month, through samples collected at the return point of the distribution loop and at one of the points in the processing (reuse) room[4].

The analytical reference methods currently available for enumerating microorganisms are widely accessible, reproducible and recognized by regulatory agencies as the gold standard[7]. Because they are based on classic cultivation methods, they depend on conditions that enable microbial growth and replication until detection by visual examination, which can take between 18 h and 14 days[5,7].

Official compendia, like the Standard Methods for the Examination of Water and Wastewater[8], recommend the use of pour plate, spread plate, and membrane filter methods for enumeration of heterotrophic bacteria and fungi in treated water for hemodialysis. However, just like in any microbiological method, the variability in cell growth is affected by the type of culture medium, by the methods used for recovery, by the condition of the microorganism during the test period and by the incubation temperature[5,9,10].

Over the years, the pour plate method has been the most commonly used for counting heterotrophic bacteria in water samples due to its low cost and easy execution. However, the addition of molten agar at approximately 45ºC-48ºC can increase stress on the microorganisms, which are already physiologically stressed, resulting in a significant decrease in bacterial recovery compared to the recovery obtained by the spread plate and membrane filtration methods[8,10,11].

The difficulty in adapting microorganisms to environments with low availability of nutrients and the inability of some species to divide and form colonies are challenges for the cultivation of microorganisms.

Until the late 1980s, culture media with high nutritional content, like soy casein agar and standard count agar, were recommended for the enumeration of heterotrophic bacteria in samples of drinking and treated water[12]. However, after the publication of the study by Reasoner and Geldreich[11], the Reasoner'2 Agar (R2A) culture medium was presented as a substitute for culture media with high nutritional content, since it enables greater bacterial recovery, especially if incubated at temperatures of 20ºC-25ºC for 5 to 7 days[5,8,9,10,11,12,13].

Low-nutrient media, such as R2A, preferably when incubated at low incubation temperatures, enable us to obtain five to ten times higher aerobic counts. This is related to the survival of bacteria under physical changes and the metabolic decline in oligotrophic environments[10,14].

Considering that the microbiological quality of the water is a critical parameter that must be monitored, it is difficult to respond to the results when the quality cannot be assessed in real time. Therefore, the time it takes to obtain the results is the main disadvantage of cultivation-based methods[14].

The excessively long time to obtain the analytical results by traditional methods published in official compendia has led to the search for alternative microbiological methods that enable the release of results in a shorter time and, thus, assist in the early investigation of failures in the water treatment[7,15,16,17]. Among them, the microbial detection technique by fluorescence to enumerate heterotrophic bacteria is an attractive option because of the possibility of reducing the analytical time, ease of conducting the assay and the precision of the results. However, the use of alternative methods depends on the demonstration of their equivalence when compared to official methods to justify their implementation to monitor the microbiological quality of treated water for hemodialysis.

The new technologies, also called rapid microbiological methods, enable the detection, identification and microbial quantification in less time when compared to traditional methods. In addition to reducing the time to release results and enabling earlier decisions based on the investigations, these technologies can offer automation of assays, greater precision, reproducibility and sensitivity[18].

However, the difficulties in evaluating, validating and obtaining approval from regulatory bodies for the use of these technologies in microbiological tests have delayed the implementation of new ways of working, since the demonstration of equivalence with official methods needs to be proven[5,17].

Official compendia like the Brazilian Pharmacopeia[19], the United States Pharmacopeia[5] and the European Pharmacopoeia[20], influenced by the publication of the PDA Technical Report n. 33, Evaluation, Validation and Implementation of new microbiological testing methods[17], recently published specific chapters to assist the scientific community and the pharmaceutical industry in the validation of alternative microbiological methods.

Methodologies that use the principle of fluorescence staining have been widely used to monitor microbial contamination in recent decades, mainly in the food industry, due to the possibility of reducing analytical time and increasing the precision of results[21].

The potential for microbial detection by fluorescence has enabled the development of analytical technologies, including the Milliflex® Quantum system, a growth-based technology that combines the membrane filtration of samples with the fluorescent staining of viable microorganisms. It is composed of a filtration pump, fluorescence reader, chamber and fluorochromes.

After the sample filtration step with the aid of a pump, the microorganisms are retained on the membrane and stained by a fluorescence viability marker. The principle of the technique is based on an enzymatic reaction in which the succinimidyl ester of carboxyfluorescein diacetate, the fluorogenic substrate, is a non-fluorescent viability marker that is cleaved by non-specific intracellular enzymes resulting in the release of the carboxy fluorescein succinimidyl ester compound (CFSE), a fluorescent product when excited at a wavelength of 488 nm[21]. Because of its low permeability to the cell membrane, the CFSE builds up inside the cells and, therefore, acts as an indicator of microbial metabolism activity and membrane integrity. This enables the detection of microcolonies after exposure to the excitation wavelength of the fluorescence dye through Milliflex® Quantum reader[21,22,23].

Since only viable cells (including endospores, vegetative forms, anaerobic bacteria and fungi) are able to retain and accumulate CFSE, these are the cells that will be identified by the fluorescent emission.

With this technique, the fluorescent microcolonies can be counted by the analyst directly through the reader of the Milliflex® Quantum equipment or through an image on a computer screen from a camera attached to the equipment reader and the use of software. With this software, one can click on the colonies and mark them as counted, thus reducing the chances of human errors.

Therefore, after a shorter incubation period, one can observe the detection of fluorescent microcolonies and release the results of enumeration of microorganisms without the need to wait for the visual count of the CFU[21,22]. This method is non-destructive and enables later identification through microbial characterization technologies, if necessary[21,22,23].

The advantages of using this system for the assessment of the microbiological quality of water treated for hemodialysis are related to the easy application of the technique and the detection of viable and cultivable microorganisms in less time, when compared to official methods, enabling the early investigation of possible failures in water treatment. Moreover, it has the advantage of being similar to traditional methods, which facilitates the performance of validation tests in view of compendial requirements, in addition to requiring lower investment for the acquisition of inputs and equipment when compared to other available technologies.

The objective of this study was to determine the minimum incubation time and evaluate the performance of the alternative microbiological method for counting heterotrophic bacteria in samples of water treated for hemodialysis using the technique of microbial detection by fluorescence.

METHOD

The study was divided into three stages: determination of the minimum incubation time (stage 1), assessment of the alternative method performance (stage 2) and equivalence (stage 3).

In all stages, the counts obtained by the fluorescence detection technique with the Milliflex® Quantum system (alternative method), as well as the counts obtained after the reincubation of the plates, also with the alternative method, were compared to those obtained with the traditional method[8]. Next, the fluorescence count recovery and viability recovery rates were calculated using the CFU log value obtained in the counts.

Traditional methodology

We used the pour plate technique with R2A (Merck, Germany) as a culture medium. The visual counting of the CFU found in the plates was performed after 120 h of incubation in a bacteriological oven at a temperature of 24.0 ± 4.0ºC[8] with the help of the colony counter.

Alternative methodology (Milliflex® Quantum)

We used the technique of filtration with membrane of mixed cellulose esters (Millipore, Germany) with a pore size of 0.45 µm and a diameter of 47 mm, using the Milliflex® Quantum system (Millipore, Germany). Plates pre-filled with R2A (Millipore, Germany) were used as culture medium for incubation at 24.0 ± 4.0ºC for the periods described in each stage of the work.

After each period, 2 mL of the fluorescence reagent (Millipore, Germany) were added to the membrane, followed by incubation for 30 min at a temperature of 32.5ºC ± 2.5°C for diffusion of the reagent. The results were recorded under the name of fluorescent count after counting the fluorescent microcolonies using the Milliflex® Quantum reader (Millipore, Germany).

After counting, the plates were reincubated in a bacteriological oven at a temperature of 24.0 ± 4.0ºC until the total incubation time of 120 h, when the CFU visual count was done with the help of the colony counter. These results were recorded under the name of viability count.

Stage 1 - Determination of the minimum incubation time by the alternative method using the Milliflex® Quantum system

We used individual inoculants acquired at a concentration of 50 CFU/100 µL of *Escherichia coli* NCTC 12923 (Biomerieux, Australia), *Staphylococcus aureus* NCTC 10788 (Biomerieux, Australia) and *Burkholderia cepacia* NCTC 10743 (Biomerieux, Australia) in order to obtain 50 CFU per plate by the traditional method. For the alternative method, 100 ml of sterile purified water artificially and individually contaminated with the same microbial inocula were filtered, in order to obtain 50 CFU per membrane.

For *Pseudomonas aeruginosa* ATCC 9027 (Microbiologics, United States of America), the preparation of the stock microbial suspension was made in soy casein broth (incubation at 32.5ºC ± 2.5°C for 24 h), followed by serial dilution of the suspension in order to obtain the final concentration of 50 CFU/mL, using 0.9% sodium chloride solution (w/v) as diluent.

The incubation periods of 24, 36, 40, 48 and 120 h were assessed for the alternative method, with the tests performed in quadruplicate for each proposed incubation time. In parallel, quadruplicate tests were performed using the traditional method.

Stage 2 - Alternative method performance evaluation

The method for enumeration of heterotrophic bacteria in water treated for hemodialysis using the microbial detection technique with fluorescence was challenged against the parameters established for quantitative tests described in the main official compendia: accuracy, precision, intermediate precision, linearity, robustness, detection limit and quantification[5,19,20].

Seven individual microbial suspensions were prepared for each of the microorganisms used in the study: *E. coli* NCTC 12923 (Biomerieux, Australia), *S. aureus* NCTC 10788 (Biomerieux, Australia), *B. cepacia* NCTC 10743 (Biomerieux, Australia) and *P. aeruginosa* ATCC 9027 (Microbiologics, United States of America). From each of these suspensions, serial dilutions were performed in order to obtain eight different concentrations between 2.5 x 10^{-1} and 1.0 x 10^{2} CFU/mL, using sodium chloride solution 0.9% (w/v) as diluent.

For the alternative method, 100 ml of purified sterile water contaminated artificially and individually with the microbial inocula were filtered, whereas for the traditional method the inocula were transferred directly to the center of the petri dishes.

The tests to assess the parameters of precision, accuracy, linearity, limits of detection and quantification were performed in duplicate for each microbial suspension, simultaneously, using the traditional method and the alternative method, together with a replica using sterile purified water as a negative control of the assay[17].

For intermediate precision studies, inoculants ranging from 10 to 100 CFU/plate were used to perform triplicate tests by analysts A and B on different days. Three replicates were tested, by concentration level for each microorganism, per analyst. The tests were conducted in duplicate for each replica, concurrently for the alternative and traditional method.

The incubation period used for the alternative method was 40 h at a temperature of 24.0 ± 4.0ºC.

Stage 3 - Equivalence

For the execution of the tests of the Equivalence stage, 24 samples of treated water for hemodialysis were collected in sterile flasks by the Municipal and State Health Surveillance groups of the state of São Paulo in dialysis services located in municipalities in the state during April and May 2019, covering 12.0% of the dialysis services in operation in the state. The treated water outlet located in the processing (reuse) room was the sampling point defined for the study. In the absence of a processing room in the unit, the return point of the distribution loop was used.

The collection and transport of samples of water treated for hemodialysis were done according to the recommendations of the American Public Health Association[8]. The material for analysis was transported in thermal boxes with a maximum capacity of 26 L (Easypatch®, São Paulo) immediately after collection, under a temperature below 10ºC, and processed on the same day of receipt[24].

The assays were performed simultaneously using the traditional and alternative methods, with incubation times of 120 h and 40 h, respectively. The test portion of the samples considered the volumes of 1 mL (10^0) and 100 µL (10^{-1}), in duplicate.

To demonstrate the non-inferiority of the alternative method in relation to the traditional method, the equivalence test (TOST) was applied to samples paired with the logarithm of the counts obtained in the assays performed in stage 2, as well as for the logarithm of the counts obtained in the assays done with the collected samples of treated water for hemodialysis.

The hypothesis that the alternative and traditional methods are equivalent was tested with the data obtained in stage 2 for the four microorganisms used in the study, as well as with the data obtained in the assays of samples collected from treated water for hemodialysis, using a lower limit of 70.0% and an upper limit of 130.0%, as recommended by international compendia[5,17].

Statistical analysis

To evaluate the results, all statistical analyses were conducted using the Minitab® v.18 software (Minitab Inc., USA).

RESULTS AND DISCUSSION

Stage 1 - Determination of the minimum incubation time by the Milliflex® Quantum system

Stage 1 intended to assess how the variation in the incubation time can affect the final result of bacterial enumeration, as well as to determine the minimum incubation time necessary to obtain reliable count values for each of the microorganisms in the study.

The results of the fluorescence and viability recoveries were calculated with the values of the counts obtained in the assays done in stage 1, after logarithmic transformation. The results are shown in Table 1.

The minimum incubation time for fluorescence detection was obtained after 36 h of incubation for *E. coli* and *S. aureus* and after 40 h for *P. aeruginosa* and *B. cepacia*, times after which recovery values greater than 90.0% were obtained for fluorescence counts.

The ANOVA test was used to assess the existence of statistical differences between the results obtained in each of the incubation periods proposed by the alternative method and the traditional method. A p-value equal to or greater than 0.05 does not reject the null hypothesis (H0), which enables us to assume that the counts obtained in the evaluated incubation times do not present statistical differences for a 95% confidence interval.

For the fluorescence counts obtained for *E. coli*, *B. cepacia* and *P. aeruginosa*, the ANOVA test indicated results greater than 0.05 for the p-values of each microorganism. This indicates that there was no significant difference between the recovery values of the incubation times we evaluated (Table 1).

However, for *S. aureus*, the ANOVA test calculated a p-value equal to 0.02 for the counts obtained by fluorescence, indicating that there was a significant difference between the recovery values of the evaluated incubation times. After applying the Tukey and Fischer tests, we found that the time that had shown some statistical difference was 36 h.

For viability counts, recovery values greater than 90.0% were obtained for all microorganisms under study, in fluorescence counts at incubation times of 24, 36, 40, 48 and 120 h, with p-value greater than 0.05 after application of the ANOVA test. With these data, we can conclude that there was no significant difference between the viability recovery values for the microorganisms under study (Table 1).

Table 1. Results of fluorescence and viability recovery using the Milliflex® Quantum system.

Microorganism	Fluorescence recovery ± SD (%)					ANOVA p-value
	24 h	36 h	40 h	48 h	120 h	
Pseudomonas aeruginosa	ND	ND	96.3 ± 2.7	97.8 ± 2.4	99.4 ± 2.1	0.42
Burkholderia cepacia	ND	ND	96.6 ± 3.4	101.0 ± 1.3	100.0 ± 0.8	0.30
Escherichia coli	ND	95.2 ± 2.5	99.7 ± 1.9	97.0 ± 4.1	95.1 ± 5.3	0.73
Staphylococcus aureus	ND	90.7 ± 1.1	100.0 ± 2.3	100.1 ± 0.9	98.3 ± 2.6	0.02

Microorganism	Recovery of viability ± SD (%)					ANOVA p-value
	24 h*	36 h*	40 h*	48 h*	120 h*	
Pseudomonas aeruginosa	98.3 ± 1.5	98.8 ± 2.7	96.5 ± 2.8	98.7 ± 2.3	99.9 ± 1.7	0.60
Burkholderia cepacia	97.5 ± 2.7	99.3 ± 2.6	96.9 ± 4.1	101.0 ± 1.0	99.5 ± 1.8	0.46
Escherichia coli	96.7 ± 1.6	98.8 ± 5.2	99.5 ± 0.6	96.6 ± 4.4	96.4 ± 5.1	0.43
Staphylococcus aureus	99.2 ± 2.1	99.9 ± 4.4	101.0 ± 3.4	99.9 ± 2.4	98.6 ± 2.7	0.82

Source: Prepared by the authors, 2019.
ND: not detected; SD: standard deviation.
* referring to the incubation time used for fluorescence detection

Therefore, the Milliflex® Quantum method proved to be robust in the range of 36 h to 120 h of incubation for *E. coli* and in the range of 40 h to 120 h for *S. aureus*, *P. aeruginosa* and *B. cepacia*.

In order to address the minimum incubation time common to all microorganisms under study, the incubation time of 40 h was selected for the performance evaluation of the alternative method through the Milliflex® Quantum system in stage 2.

Stage 2 - Performance evaluation

The counting results obtained by the traditional method were used as expected values for the standard inoculum, at their respective concentration levels.

To evaluate the accuracy of the alternative method, the results of fluorescence and viability recoveries were calculated using the logarithm of the values of the counts obtained in the assays done in stage 2.

The fluorescence and viability recovery values were higher than 90.0% for the concentration levels between 5 and 100 CFU/plate for *S. aureus*, *E. coli*, *B. cepacia* and *P. aeruginosa* with a maximum standard deviation value of 24.5% for fluorescence recovery with the 5 CFU/plate inoculum referring to *S. aureus*.

No microbial growth was obtained for the inocula with a target concentration of 0.25 and 0.5 CFU/plate using the alternative and traditional methods for *B. cepacia*, *E. coli* and *P. aeruginosa*.

For the inoculants with a target concentration of 1 CFU/plate of all microorganisms under study, as well as for the inoculum with a target concentration of 0.5 CFU/plate of *S. aureus*, no microbial growth was obtained in none of the replicas tested by alternative and traditional methods.

Therefore, since some replicas presented the mean count value equal to one or zero, after logarithmic transformation, these data resulted in values equal to zero, making it impossible to calculate the mean value of fluorescence recovery and viability for the alternative method for the inocula with a target concentration of 0.5 CFU/plate and 1 CFU/plate.

Additionally, Levene's test was applied to assess the homogeneity of variance by concentration range for each microorganism, for the 95% confidence interval. P-value results greater than 0.05 were obtained for the range of 5 to 100 CFU/plate, indicating that the errors present homogeneous variance, which is a condition for the application of the ANOVA test.

Then, the one-way ANOVA test was used for the 95% confidence interval, with the objective of determining whether the logarithm of fluorescence and viability counts obtained by the alternative method after 40 h and 120 h of incubation, respectively, was statistically different from the logarithm of the counts obtained by the traditional method after 120 h of incubation.

A p-value equal to or greater than 0.05 does not reject the null hypothesis (H0), which enables us to assume that the logarithm of the counts obtained by the alternative method (fluorescence and viability) and by the traditional method do not present statistical differences for a 95% confidence interval.

The one-way ANOVA test indicated results greater than 0.05 for the p-values related to each concentration level of each microorganism. This suggests that there is no statistical difference between the logarithm of fluorescence and viability counts obtained by the alternative method and by the traditional method for the range of 5 to 100 CFU/plate for all microorganisms under study.

To evaluate the precision, the coefficients of variation (CV) were calculated for the logarithm of the counts by fluorescence, viability and for the logarithm of the counts obtained by the traditional method for all microorganisms, at each concentration level.

According to the Parenteral Drug Association[17], coefficients of variation of less than 35.0% are accepted for traditional plate counting methods for counts greater than 10 CFU/plate. Meeting this criterion with the alternative method eliminates the need to compare the coefficient of variation of the alternative method with the coefficient of variation of the traditional method[17]. The Brazilian Pharmacopoeia[19] establishes that values below 30.0% for the coefficient of variation demonstrate acceptable precision for the methods.

As expected, the highest values of coefficient of variation were obtained for the counts done with inocula with a target concentration of 5 CFU/plate, for the four microorganisms under study, as shown in Table 2. For 10, 25, 50 and 100 CFU/plate inoculants, the CV obtained are also in compliance with the normative acceptance criteria.

For studies on intermediate precision, the CV were calculated for the logarithm of the counts by fluorescence, viability and the traditional method for all microorganisms, at each concentration level.

For the following microorganisms, *S. aureus*, *E. coli*, *B. cepacia* and *P. aeruginosa*, the highest values of coefficient of variation were obtained for the logarithm of the counts related to the inoculum of 10 CFU/mL (Table 2), with the exception of the coefficient of variation calculated with the viability counts for the inoculum of the 25 CFU/plate of *B. cepacia*, which presented a value of 2.7%, higher than that of the 10 CFU/plate (1.6%). All counts obtained with the inocula of concentration between 10 and 100 CFU/plate presented CV below 30.0%.

In order to assess whether the variability of measurements could be explained by the difference in analysts, a repeatability and reproducibility (R&R) study was carried out. For conducting the test, the logarithmic values of fluorescence and viability counts obtained by the alternative method were used, as well as the visual counts obtained by the traditional method, by analysts A and B.

Table 2. Coefficients of variation referring to the precision (n = 7) and intermediate precision (n = 6) assays for the 5 CFU/plate and 10 CFU/plate inocula, respectively.

Microorganism	Coefficient of variation (%) - Precision		
	Fluorescence	Viability	Traditional method
S. aureus	19.8	21.1	28.7
E. coli	14.7	13.4	14.8
B. cepacia	11.2	13.7	11.8
P. aeruginosa	8.9	8.2	9.2

Microorganism	Coefficient of variation (%) - Intermediate precision		
	Fluorescence	Viability	Traditional method
S. aureus	4.3	3.8	4.3
E. coli	6.2	4.5	5.4
B. cepacia	3.4	1.6	3.9
P. aeruginosa	3.8	4.4	5.7

Source: Prepared by the authors, 2019.

The evaluation of homogeneity of variances using Levene's test between the groups of fluorescence and viability counts and traditional method for each microorganism, with a 95% confidence interval, demonstrated that the data present homogeneity of variances (p-value greater than 0.05), an assumption for the application of the ANOVA gauge, contemplated in the R&R study.

The ANOVA tables of the R&R studies indicated results greater than 0.05 for the p-values related to each microorganism. This indicates that the interaction between day and analyst was not significant at the level of significance of 5% for the counts obtained by the alternative method, (fluorescence and viability) and the traditional method (data not shown).

The evaluation tables of the measurements of the R&R studies for the four microorganisms under study presented values between 3.7% and 7.4% for the percentage of variation of the study and minimum value of 19 for the number of distinct categories (*S. aureus*). Both these parameters are in accordance with the determined acceptance criteria: coefficient of variation of the study below 30.0% and number of different categories above 5.

The sample range obtained by analysts A and B for each microorganism, by level of concentration and methodology, is demonstrated by the R control charts in Figure 1.

As can be seen in Figure 1, all points are within the limits of the sample range control chart, thus demonstrating that the measurements between analysts are consistent.

For the study of linearity with the microorganisms under study, Pearson's correlation test indicated a strong correlation (values greater than 0.95) between the fluorescence and viability counts obtained by the alternative method compared to the counts obtained by the traditional method. A p-value below 0.05 for both correlations enables us to reject the hypothesis that the correlation coefficient is equal to zero, indicating that there is a significant relationship between the tested variables.

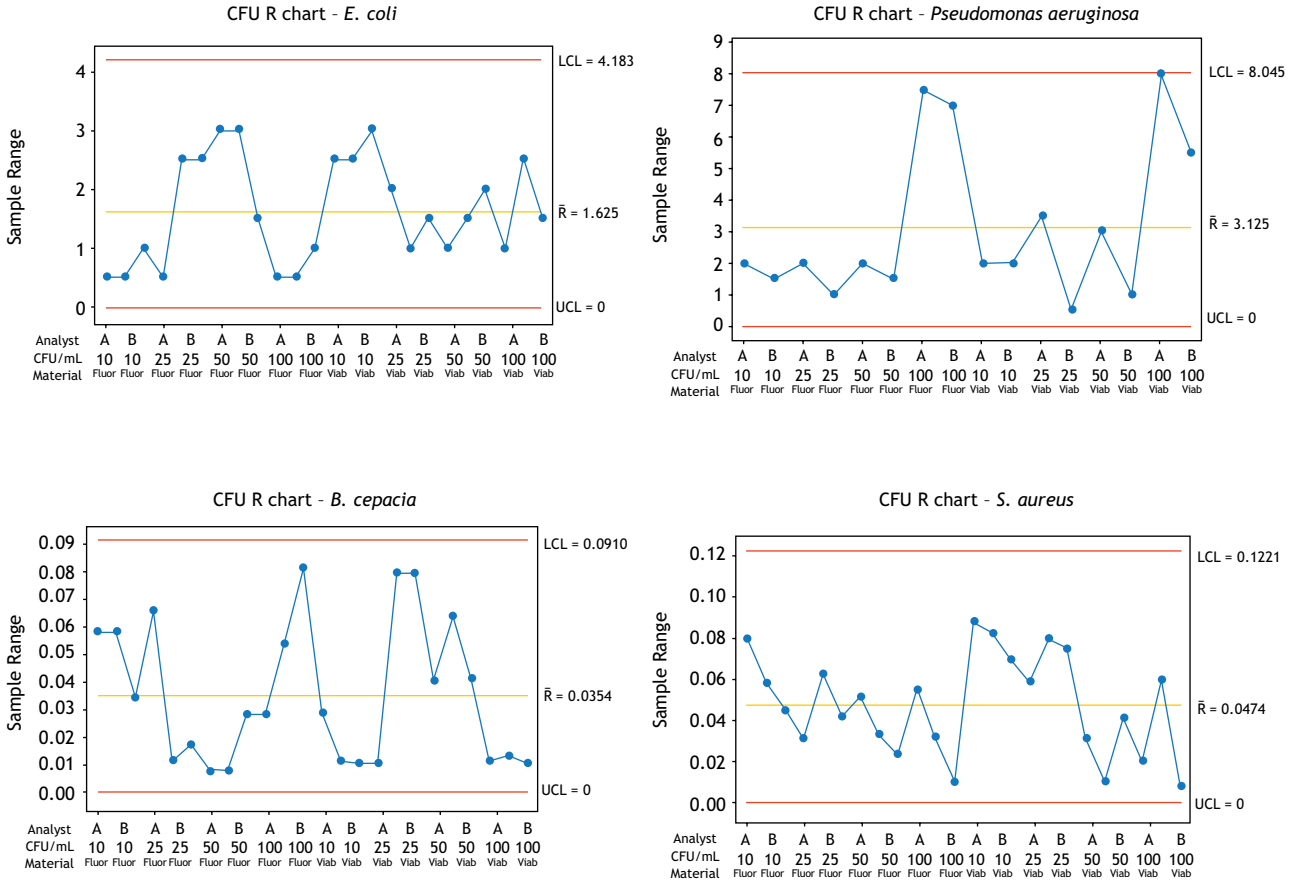
For the individual linear regression analyses of each microorganism under study, we used the mean of the logarithms of the counts obtained by the seven replicates by concentration level with the alternative method (fluorescence and viability) and with the traditional method. For the linear regression analyses addressing the data set of the four microorganisms, we used the mean of the logarithms of the counts obtained by the seven replicates by concentration level with the alternative method (fluorescence and viability) and with the traditional method of each microorganism.

The regression analysis has shown a linear correlation between the fluorescence and viability counts obtained by the alternative method compared to the counts obtained by the traditional method for the range of 5 to 100 CFU/plate, for all microorganisms under study, as can be seen in Figure 2.

The residual plots obtained in the individual linear regression analyses for each microorganism, as well as in the analyses that included the data set of the four target microorganisms, were evaluated for the normality of the standardized residuals, variance and independence of the residuals.

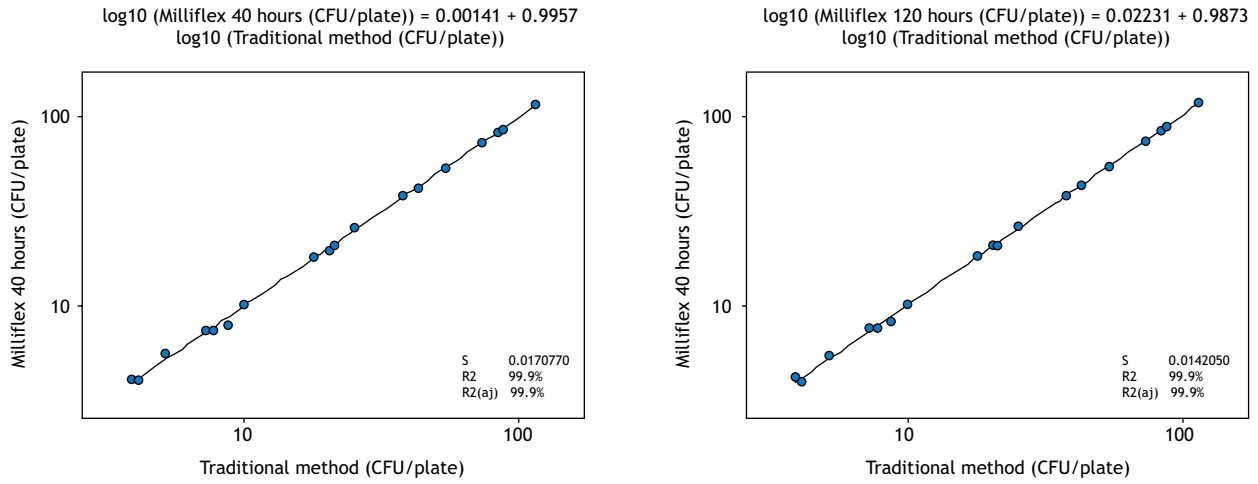
The presumption of normality in the residual distribution was confirmed by a p-value greater than 0.05 for the range between 5 CFU/plate and 100 CFU/plate, for each microorganism, through the normality test and the normal probability plot of the residuals. With these values, the alternative hypothesis can be rejected, allowing us to assume that the residual distribution is normal.

The residual plots versus adjustments (Figure 3) enabled us to verify the assumption that the residuals are randomly scattered and present constant variance. This hypothesis was confirmed after application of Levene's test to assess equality of variances, by concentration range, in which results of p-value greater than 0.05 were obtained for the range of 5 to 100 CFU/plate. With these values, the alternative hypothesis can be rejected, allowing us to assume that the residual errors are homoscedastic.



Source: Prepared by the authors, 2019.

Figure 1. R chart of the repeatability and reproducibility study for the microorganisms under study.

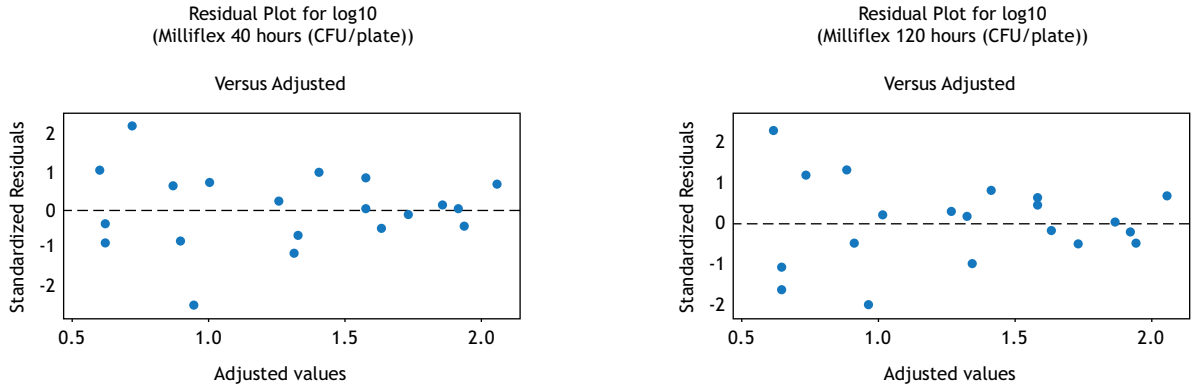


Source: Prepared by the authors, 2019.

Figure 2. Linear regression analysis including *S. aureus*, *E. coli*, *P. aeruginosa* and *B. cepacia*: fluorescence vs. traditional method; viability vs. traditional method.

The independence of the residuals was observed through the residual plot versus order generated by the Minitab software.

Therefore, by performing the linear regression analysis of the data obtained in the range of 5 to 100 CFU/plate for the four challenged microorganisms, we could assess the existence of a correlation between fluorescence counts (alternative method) and visual counts (traditional method), as well as between viability counts (alternative method) and visual counts (traditional method).



Source: Prepared by the authors, 2019.

Figure 3. Residual plot *versus* adjustments including *S. aureus*, *E. coli*, *P. aeruginosa* and *B. cepacia*: fluorescence vs. traditional method; viability vs. traditional method.

The specificity of the alternative method was evidenced by the positive detection of Gram-positive (*S. aureus*) and Gram-negative (*E. coli*, *B. cepacia and P. aeruginosa*) bacteria, challenged for the range of 5 to 100 CFU/plate.

For the detection limit and quantification tests, no microbial growth was observed in the plates inoculated with bacterial suspensions with a target concentration of 0.25 and 0.5 CFU/plate by the alternative and traditional methods for *B cepacia, E. coli* and *P. aeruginosa*.

For *E. coli*, *B. cepacia* and *P. aeruginosa*, it was possible to observe microbial growth in at least 50.0% of the plates inoculated with suspension at the target concentration of 1 CFU/plate using the alternative and traditional methods, whereas for *S. aureus*, this observation was made from the suspension with a target concentration of 5 CFU/plate.

Therefore, for the limit of detection parameter, the concentration range in which microbial growth was observed in 50.0% of the samples by the traditional method for all challenged microorganisms was 5 CFU/plate, with positive detection in all replicas. The same was verified in the alternative method for fluorescence and viability counts. For this range, Student's T test did not identify statistical differences between fluorescence counts (alternative method) and visual counts by the traditional method as well as between viability counts (alternative method) and visual counts by the traditional method (p-value greater than 0.05), for the four microorganisms under study.

For the limit of quantification parameter, the lowest concentration tested that met the criteria of precision, accuracy and linearity for all microorganisms evaluated for the alternative method was 5 CFU/plate. Therefore, the limit of quantification of 5 CFU/plate was established for the alternative method.

To determine the robustness of the alternative method, the sample incubation time parameter was evaluated. The assays from stage 1 were used to evaluate the results.

Stage 3 - Equivalence

Equivalence studies are used to demonstrate the absence of differences between the results obtained by two analytical methods, while non-inferiority studies aim to demonstrate that a new analytical method is not, according to certain criteria, less sensitive, accurate or precise than another existing analytical method. Thus, the formulation of the hypotheses to be tested is what characterizes the difference between the two types of studies.

In assessing the performance of the alternative method, the non-inferiority of the alternative method in relation to the traditional method needs to be proven[19,20].

To that end, the TOST was applied to samples paired with the logarithm of the counts obtained in the assays performed in stage 2, as well as for the logarithm of the counts obtained in assays done with the collected samples of treated water for hemodialysis described in stage 3.

Regarding the data obtained in the assays performed with the 24 samples of water treated for hemodialysis in stage 3, the results of the samples that presented heterotrophic bacteria count equal to zero (n = 9) were excluded from the statistical analysis in this stage due to the impossibility of a statistical comparison for equivalence between the alternative method and the traditional method if both methods report absence of microbial growth. One sample also had its results excluded from the statistical analysis at this stage due to the impossibility of quantification, since the counts obtained on the 10^{-1} dilution plates were greater than 100 CFU/plate, resulting in values greater than 1,000 CFU/mL for this sample. Thus, the statistical analyses included the evaluation of 14 samples (n = 14).

The null hypothesis that the alternative method is not inferior to the traditional method was tested using a lower limit of 70.0%.

Rejecting the null hypothesis means that the difference between the numerical result of the traditionally employed methodology and that of an alternative methodology is within the margin of tolerance and, therefore, the alternative method is not inferior to the traditional method. The alternative method is considered to be non-inferior if the lower limit of the 95% confidence interval of the difference between traditional and alternative method does not include the specified margin value. Thus, a p-value below 0.05 as a result of the test allows the rejection of the null hypothesis and proves the non-inferiority of the alternative method compared to the traditional method, for a 95% confidence interval.

For all microorganisms tested in stage 2 as well as for the hemodialysis water samples evaluated in stage 3, the count values obtained are within the limits of non-inferiority, demonstrating the non-inferiority of the alternative method when compared to the traditional method (data not shown).

The equivalence test with paired data can be used to test whether the mean of a test data set is equivalent to the mean of a reference data set when observations are made concurrently. The test equivalence is determined by an interval of specified values called equivalence interval. The result will determine whether the evidence is sufficient to state that the ratio between the means of the data sets is within the equivalence interval.

The hypothesis that the alternative and traditional methods are equivalent was tested with the data obtained in stage 2 for the four microorganisms used in the study, as well as with the data obtained in the tests of samples collected from treated water for hemodialysis in stage 3, with a lower limit of 70.0% and an upper limit of 130%.

A p-value of less than α (0.05) allows us to reject the null hypothesis and conclude that the difference between the means is included in the equivalence interval, and that the means are therefore equivalent.

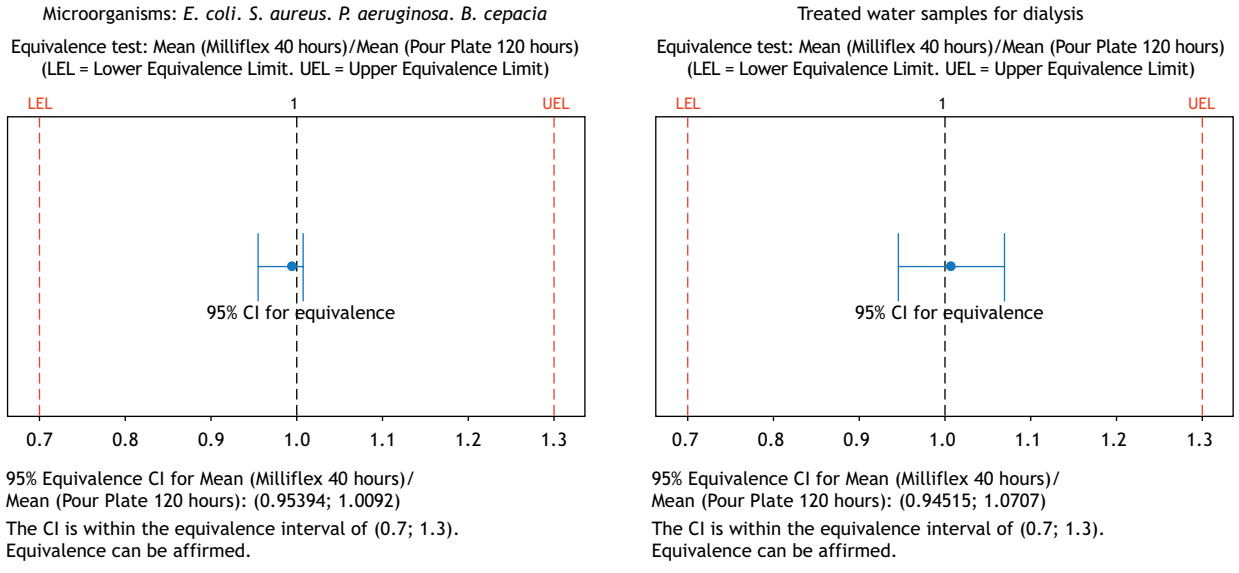
According to what we see in Figure 4, it is possible to state that the alternative method, for a minimum incubation period of 40 h, achieves results that are equivalent to those of the traditional method, since the confidence interval of the alternative method is fully within the equivalence interval in both graphs.

CONCLUSIONS

The results have shown that the alternative method achieves results equivalent to the traditional method, enabling the quantification of heterotrophic bacteria after 40 h of incubation with accuracy, precision, specificity and linearity for the range of 5 to 100 CFU/plate, resulting in a reduction of approximately 67.0% of the time currently required in the traditional method.

Therefore, from these results we can draw the conclusion that the alternative methodology was linear and specific for the enumeration of *S. aureus, E. coli, B. cepacia and P. aeruginosa,* since it was possible to positively detect all the target microorganisms, as well as correlation coefficients greater than 0.95 and straight lines with a slope between 0.8-1.2 were obtained.

Based on the statistical evaluations presented here, it is possible to conclude that the effects of the day and analyst factors were not significant for the study of intermediate precision for all microorganisms under study.



Source: Prepared by the authors, 2019.

Figure 4. Equivalence test between the counts obtained by the alternative and traditional method in stages 2 and 3, including microorganisms and samples of treated water for hemodialysis.

The alternative methodology proved to be precise for all microorganisms under study, since the coefficient of variation values obtained are below 30.0%, the most restrictive criterion, for the range of 5 to 100 CFU/plate.

The alternative method proved to be robust for the period between 40 h and 120 h of incubation using the Milliflex® Quantum system for all microorganisms under study.

Furthermore, it is possible to state that the alternative method, for a minimum incubation period of 40 h, achieves results that are equivalent to those of the traditional method, since the confidence interval of the alternative method is fully within the equivalence interval.

Therefore, the microbial detection technique using fluorescence is a feasible option for the setup of a rapid microbiological method for counting heterotrophic bacteria in samples of water treated for hemodialysis.

The importance of implementing rapid microbiological methods for monitoring the microbiological quality of treated water for hemodialysis is an important tool to help public health laboratories reduce the risks to which chronic kidney patients in hemodialysis are exposed.

With the results obtained with the alternative method in this study, we recommend using this methodology to increase the analytical response capacity of the public and private service providers in charge of the quality control of treated water for hemodialysis in order to improve health regulatory actions in health services.

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Authors' Contribution

Hilinski EG, Bugno A - Conception, planning (study design), acquisition, analysis, interpretation of data and writing of the paper. Silva FPL, Almodovar AAB, Pinto, TJA - Analysis, interpretation of data and writing of the paper. All authors approved the final draft of the paper.

Conflict of interest

The authors report that there is no potential conflict of interest with peers and institutions, nor political or financial conflicts in this study.