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Original

Reliability of quantification of Candida species colonies using alternative plating methods

Confiabilidade da quantificação de colônias de espécies de Candida utilizando métodos alternativos de plaqueamento

Fiabilidad de la cuantificación de colonias de especies de Candida utilizando métodos alternativos de enchapado

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ABSTRACT

Objective: to evaluate the concordance of different plating methods for quantification of *Candida* species colonies. **Method**: standardized suspensions of reference strains (*Candida albicans, Candida glabrata, Candida tropicalis* and *Candida krusei*) were submitted to serial dilution and plating according to methods of track-dilution (TDM), drop plate (DPM) and the conventional spread plate (SPM). Data were submitted to construction of Bland–Altman diagrams, Intraclass Correlation Coefficient (ICC) and ANOVA (α=5%). **Results**: adequate concordance between the methods (CCI >0.71) was observed, and the execution of DPM was the fastest ($p<0.001$). However, DPM and TDM appear to result in greater values compared to SPM, especially for *C. tropicalis* and *C. krusei*. **Conclusion**: *C. albicans* and *C. glabrata* can be plated with DPM and TDM, but the use of these methods for *C. krusei* and *C. tropicalis* may result in count variation.

Descriptors: *Candida albicans*; *Candida tropicalis*; *Candida glabrata*; Data accuracy; Colony Count, Microbial.

RESUMO

Objetivo: avaliar a concordância de diferentes métodos de plaqueamento para a quantificação de colônias de espécies de *Candida*. **Método**: suspensões padronizadas das cepas de referência (*Candida albicans, Candida glabrata, Candida tropicalis* e *Candida krusei*) foram submetidas à diluição seriada e plaqueamento segundo os métodos plaqueamento em rastro (MPR), plaqueamento por gota (MPG) e o convencional espalhamento em placas (MEP). Os dados foram submetidos à construção de diagramas Bland–Altman, Coeficiente de Correlação Intraclasse (CCI) e ANOVA (α=5%). **Resultados**: foi observada adequada concordância entre os métodos (CCI >0,71), e a execução do MPG foi a mais rápida ($p<0,001$). No entanto, o MPG e MPR parecem resultar em valores superiores em relação ao MEP, especialmente para *C. tropicalis* e *C. krusei*. **Conclusão**: *C. albicans* e *C. glabrata* podem ser plaqueadas com MPG e MPR, mas a utilização desses métodos para *C. krusei* e *C. tropicalis* podem resultar em variação na contagem.

Descritores: *Candida albicans*; *Candida tropicalis*; *Candida glabrata*; Confiabilidade dos Dados; Contagem de Colônia Microbiana.

RESUMÉN

Objetivo: evaluar la concordancia de diferentes métodos de recubrimiento para la cuantificación de colonias de especies de *Candida*. **Método**: las suspensiones estandarizadas de las cepas de referencia (*Candida albicans*, *Candida glabrata*, *Candida tropicalis* y *Candida krusei*) se sometieron a dilución en serie y recubrimiento de acuerdo con el recubrimiento de rastro (MRR), sembrado por gota (MSG) y los métodos de siembra convencionales (MEP). Los datos se sometieron a la construcción de diagramas de Bland–Altman, Coeficiente de Correlación Intraclase (CCI) y ANOVA (α=5%). **Resultados**: se observó un acuerdo adecuado entre los métodos (CCI>0,71), y la ejecución de la MSG fue la más rápida ($p<0,001$). Sin embargo, MSG y MRR parecen dar lugar a valores más altos que MEP, especialmente para *C. tropicalis* y *C. krusei*. **Conclusión**: *C. albicans* y *C. glabrata* se pueden colocar en placas con MSG y MRR, pero el uso de estos métodos para *C. krusei* y *C. tropicalis* puede dar lugar a variaciones en el recuento.

Descriptores: *Candida albicans*; *Candida tropicalis*; *Candida glabrata*; Exactitud de los Datos; Recuento de Colonia Microbiana.

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INTRODUCTION

Genus *Candida* consists of heterogeneous fungal species, and some of them can act as opportunistic pathogens of humans. *Candida albicans* is considered the most prevalent species among infections,[1] but other species such as *Candida glabrata*, *Candida tropicalis* and *Candida krusei* have often been isolated.[2-4] The infections by *Candida* known as oral candidosis and oropharyngeal candidosis are considered the most frequent fungal pathologies among humans.[1] Among immunocompromised individuals, the local infections may lead to systemic damage, in which the number of deaths as a result of infection by *Candida* spp. may vary between 40% to 80%.[5-7]

The search for new effective therapies in inactivation of *Candida* species and in reducing its ability to adhere to substrates such as dental prostheses, without causing collateral effects and/or antifungal resistance, is an important field of scientific investigations.[8-9] In these studies, the growth and subsequent colony count in Petri plates has been a common practice in methodologies that seek to assess the effectiveness of new antimicrobial treatments.[10]

The conventional method used for enumeration of colony forming units per milliliter (CFU/mL) of *Candida* species is denominated spread plating (SPM).[11] The enumeration by SPM begins with the sample serial dilution, followed by transferring aliquots of samples diluted to surface of a previously solidified agar and, with the help of a plastic, glass or steel spatula, the aliquot is uniformly distributed over the culture medium.[12] After the incubation of plates for determined periods and temperature, the colonies are counted and, then, the cell concentration in original sample can be estimated referencing the volume of plate sample.

To increase the estimate precision of the number of CFU/mL in samples, aliquots of each dilution are plated in duplicate or triplicate, and the colonies are counted only in plates referring to dilution that generated a number of colonies between 30 and 300.[11,13] As the real concentration of microorganisms present in a sample after conclusion of an experiment is unknown, samples are often diluted up to seven times from the initial sample and only the replicates of a single dilution will be effectively used for count.[13] Thus, this technique, although relatively accurate and widely used, has drawbacks such as the time spent to prepare materials and plating of samples, and the great amount of disposable plastics and culture media necessary for its proper performance.[12,14]

Alternative plating methods, in which it is possible to reduce material volume and time spent for its execution, have been proposed in literature,[15-17] such as drop plating (DPM),[11,13,18] spiral plating[19] and track-dilution (TDM).[12] The last one was initially proposed for the quantification of bacteria *Enterococcus faecalis*, *Staphylococcus aureus*, *Bacillus subtilis* and *Escherichia coli* plating in culture medium Brain Heart Infusion (BHI) and showed CFU/mL values statistically similar to the conventional SPM for selected species.[12] TDM has significant advantages over SPM, such as its execution simplicity, shorter time required to prepare plates, plating and colony count and smaller volume of utilized material (plates and culture medium). That is probably why TDM has been

widely used in applied microbiology studies,[20-21] including in dentistry,[22] and for quantification of yeast[23] and *Candida* species.[9]

Although TDM is widely used, reports that have previously verified its reliability and validity for plating and quantification of *Candida* species are not found. The measure accuracy question in laboratory studies is more relevant today than ever. Results obtained in a biased way, without previous assessment of precision and reliability of performed measures, may lead to incorrect results and release of erroneous information that, consequently, provoke the lack of reproducibility in science and even adoption of ineffective antimicrobial treatments in subsequent experiments.[24]

When different plating methods are used or when different researchers apply the same method, variations in microorganism count often occur.[11] When neglected, such variations may negatively affect the study conclusions and make more difficult the comparison of results from different authors, especially those that seek to assess the effectiveness of antimicrobial therapies. Data published in literature already showed the importance of proposing standardized procedures and verifying reliability and reproducibility of plating method, in order to obtain reliable results, when it is about microbial quantification. However, such studies were conducted only for bacterial species.[11,13]

A previously published study[25] already showed that obtaining high correlation between two plating methods was dependent on *Candida* species since they can differ in colony shape and size. The same authors also emphasized that the applicability of a new method for *Candida* enumeration depends on its ability to estimate CFU/mL values from samples with high or low cell concentration. Moreover, the detection limit of each method should be considered.[12] In this context, the objective of this study was to investigate TDM and DPM reliability for quantification of the number of CFU/mL of different *Candida* species from samples with different cell concentrations and compare the time needed for execution of each method.

METHOD

Experimental outline

Experimental study performed in laboratory. The response variables investigated were CFU/mL and procedure time (in minutes). As independent variables, the plating method (Spread plate method – SPM, Track-dilution method – TDM and Drop plate method – DPM) was considered.

Strains and cultivation conditions

Reference strains from company American Type Culture Collection (ATCC), from species *Candida albicans* (ATCC 90028), *Candida glabrata* (ATCC 2001), *Candida tropicalis* (ATCC 4563) and *Candida krusei* (ATCC 6258) were used. The microorganisms were kept under freezing (−70 degrees Celsius) until the moment of its usage in Yeast-Peptone-Glucose (Sigma Aldrich Co Ltd, Missouri, USA).

To obtain cell suspensions, experimental procedures of cell incubation, wash and resuspension were standardized.[26] The cells were resuspended in 3mL of phosphate-buffered saline (PBS) and 10-fold dilutions were performed. The samples were named D0 (initial suspension) to D5 (last dilution). Samples D0 to D5 were obtained in

a standardized way on all occasions of the experiment, until they totaled 30 repetitions (n=30).

Sample plating

Each sample was serially diluted in 96-well plates[27] (Kasvi, Paraná, Brazil) and aliquots of these dilutions were plated, in duplicate, in Petri plates containing Sabouraud Dextrose Agar medium with chloramphenicol (SDA; Acumedia Manufactures, Michigan, USA). The plating for later enumeration of the number of colonies was performed according to three different proposed methods: TDM, DPM and SPM.

For SPM, two 100µL aliquots of each dilution were individually transferred to two flat-surface round Petri plates (90x15mm; Kasvi, Paraná, Brazil) containing the previously solidified SDA medium. With the aid of a L-shaped sterile glass rod, the aliquots were evenly spread over the whole culture medium surface. For TDM, 10µL aliquots of serial dilutions were transferred, with the help of a multichannel pipette (Eppendorf, Hamburg, Germany) for two square Petri plates (100x15mm, 13mm grid; Simport Scientific, Canada) containing the previously solidified SDA medium. After transferring aliquots, the plates were inclined at an angle of approximately 45° to the workbench surface, allowing that the aliquots formed tracks on the culture medium. In this method, it was possible to plate all samples (D0 to D5) in a same plate, and a second plate was used for the duplicates. When the track formed by any of the aliquots reached the distance of 5mm from agar edge, the plate was slightly tilted back to avoid aggregation of tracks at the plate base. For DPM, 10µL aliquots in duplicate from serial dilutions were transferred, with the aid of a multichannel pipette, to Petri plates containing the previously solidified SDA medium.

For all the methods, the plates were let on the workbench for 10 minutes and then, inverted and incubated at 37°C. For SPM, the incubation time used was 48h, which enables the colony growth in proper size to avoid their joining. Nevertheless, in TDM and DPM, the colony growth area was much smaller and thus, the incubation time of 20h and 18h was defined, respectively.

After the incubation period, all the plates were photographed on a black background, in a standardized way, and the colony count was performed in photographs, with the help of the Count tool in Adobe Photoshop (Adobe Systems Incorporated, San Jose, CA, USA) software, by two previously and properly calibrated researchers (CCI=0.995). For SPM, the plating volume was 0.1mL, and the count was performed in the dilution that presented growth between 30 and 300 colonies, considering the count pattern in literature. As in TDM and in DPM, the plating volume was 0.01mL, the growth pattern does not enable number of countable colonies above 30 colonies. Therefore, the count was performed in dilution that presented between 3–30 colonies because the plating volume in these methods was 10 times smaller than the plating volume in SPM. The time (in minutes) needed for plating (excluding the procedure of serial dilution) and colony count was measured with the aid of a digital stopwatch (Kasvi, Paraná, Brazil), by an independent researcher.

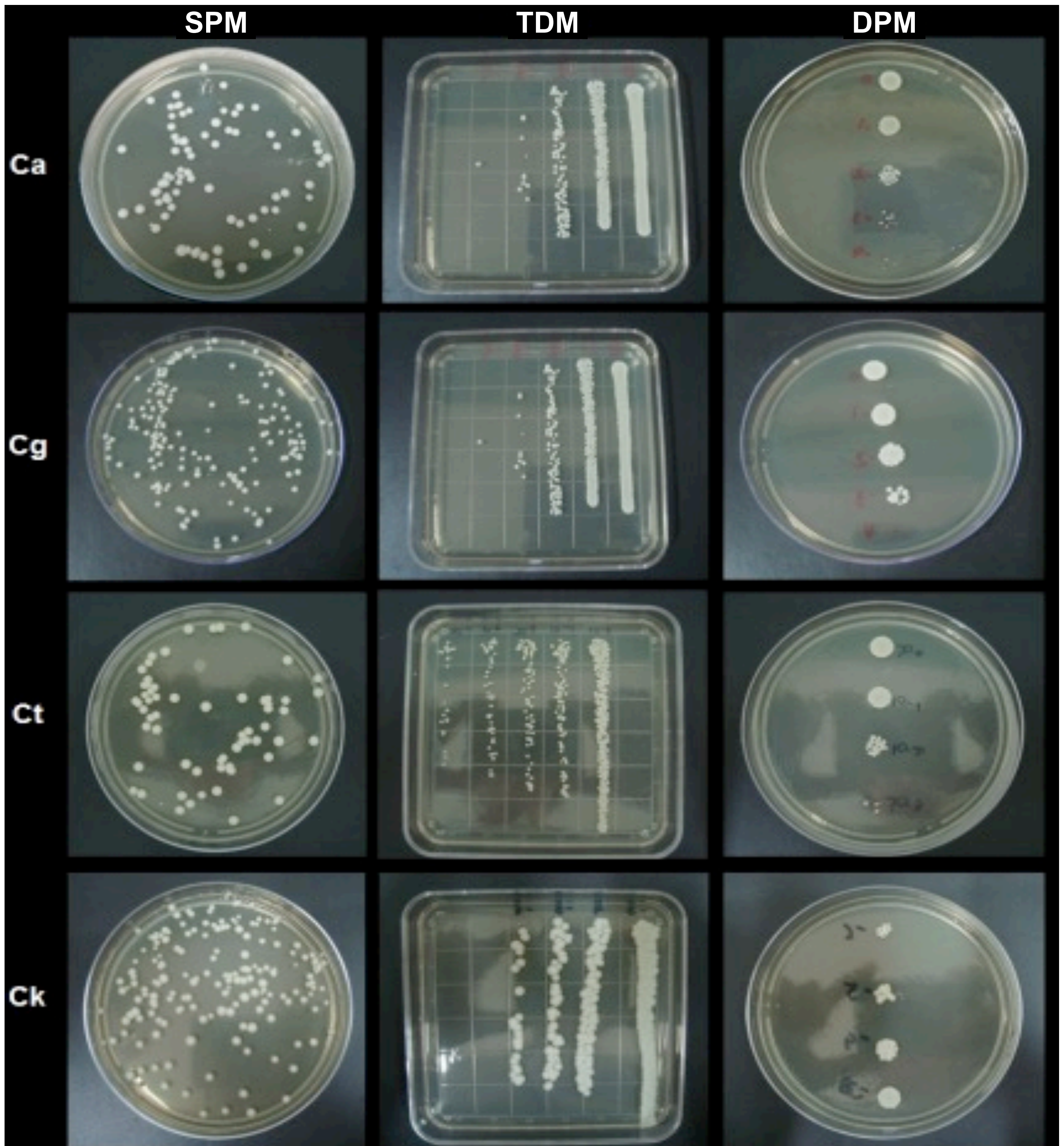
Data analysis

The values of CFU/mL were transformed in $\log_{10}$. The reproducibility between the methods was estimated through Intraclass Correlation Coefficient (ICC) and its Confidence Interval ($CI_{95\%}$), considering the absolute concordance and the two-way mixed effect model analysis of variance, for average measurements.[28] Depending on the ICC value obtained, the concordance rate between the measures was classified according to Fermanian proposal.[29] The effect of the plating type over the $\log_{10}$(CFU/mL) variable was also analyzed through repeated measures analysis of variance. The analysis of plating procedure times and count used one-way analysis of variance with Welch correction, followed by Games–Howell post-hoc test. The null hypothesis of this study was that there is no effect from different plating methods and from different species in number of colonies recovered after plating and in method execution time. Significance level was set at 5% in all analyses, which are performed in IBM Statistical Package for the Social Sciences (SPSS) Statistics 21 software.

RESULTS

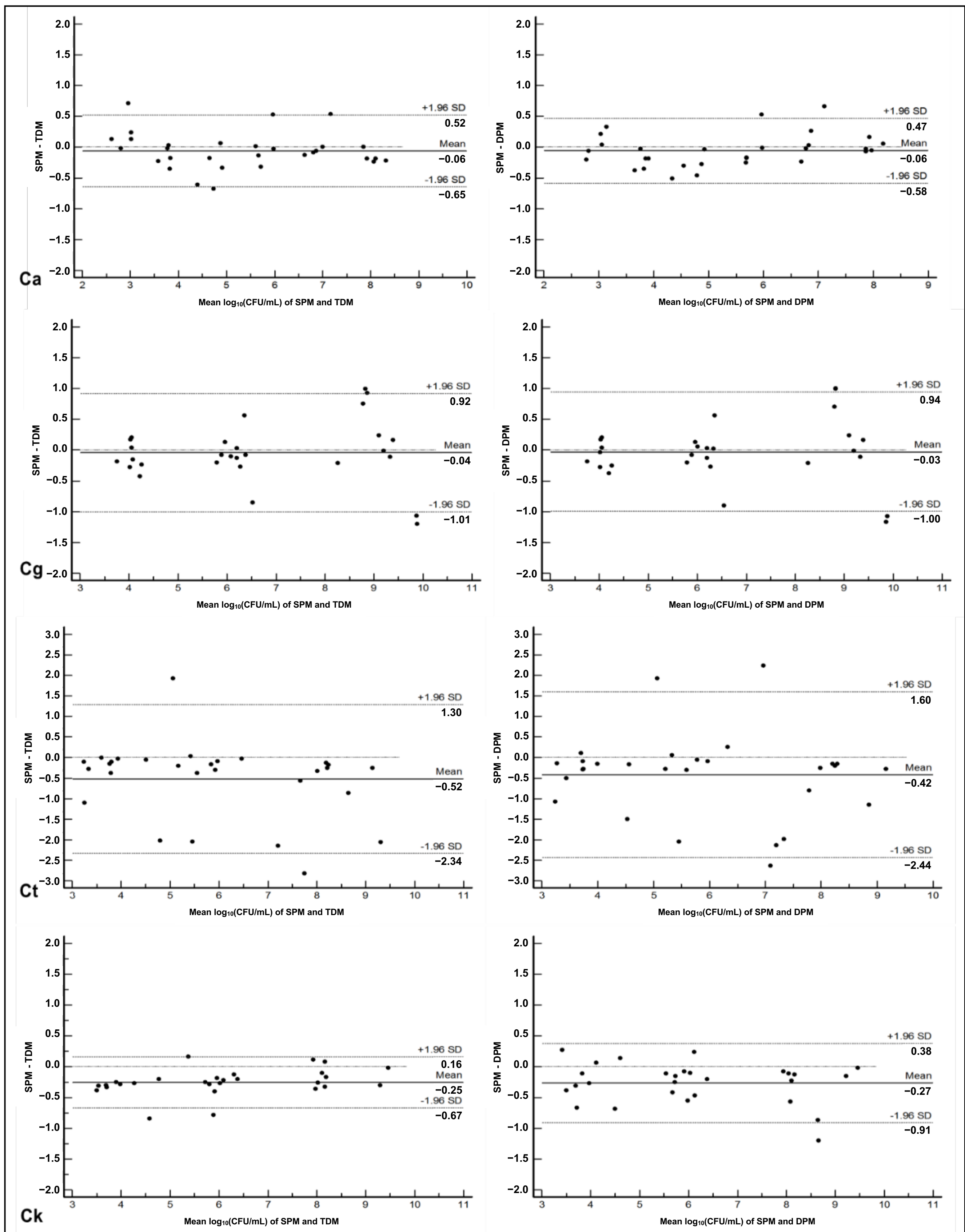
In this study, the three plating methods were compared regarding their concordance to obtain CFU/mL values (Figure 1). Descriptive data analysis pointed out that, in a general way, both alternative methods (Track-dilution method – TDM and Drop plate method – DPM) seemed to result, systematically, in $\log_{10}$(CFU/mL) values slightly higher in relation to traditional method (Spread plate method – SPM) (Table 1). Bland-Altman[30] diagrams (Figure 2) corroborated this behavior, especially for *Candida tropicalis* and *Candida krusei* species since most of the differences between $\log_{10}$(CFU/mL) values (SPM − TDM and SPM − DPM) were negative.

Figure 1: Petri plates images containing *Candida* colonies.



Caption: SPM: Spread plate method; TDM: Track-dilution method; DPM: Drop plate method; Ca: *Candida albicans*; Cg: *Candida glabrata*; Ct: *Candida tropicalis*; Ck: *Candida krusei*.

Figure 2: Bland–Altman diagrams for concordance descriptive analysis between the plating methods according to *Candida* species.



Caption: SPM: Spread plate method; TDM: Track-dilution method; DPM: Drop plate method; x axis: value mean of each method with the SPM values; y axis: difference between each method and SPM.

The difference between means was also investigated and considered statistically significant for *C. krusei* species. Bonferroni post-test pointed out smaller values in SPM compared to TDM and DPM ($p \leq 0.001$), and the last ones were considered similar to each other ($p=1.000$). Nevertheless, the reproducibility analysis pointed out an adequate concordance between methods because all ICC values were above the value of 0.71 (Table 2).

Table 1: $\log_{10}$(CFU/mL) mean values (and standard deviation) obtained after plating colonies of four *Candida* species using different plating methods.

Species	SPM		TDM		DPM		p*
Candida albicans	5.32	(1.80)	5.38	(1.81)	5.38	(1.70)	0.333
Candida glabrata	6.61	(2.12)	6.64	(2.11)	6.63	(2.11)	0.696
Candida krusei	5.97	(1.85)	6.23	(1.80)	6.24	(1.91)	<0.001
Candida tropicalis	5.71	(1.90)	6.17	(2.10)	6.07	(1.97)	0.079

Caption: *p-values obtained from repeated measures analysis of variance. SPM: Spread plate method; TDM: Track-dilution method; DPM: Drop plate method.

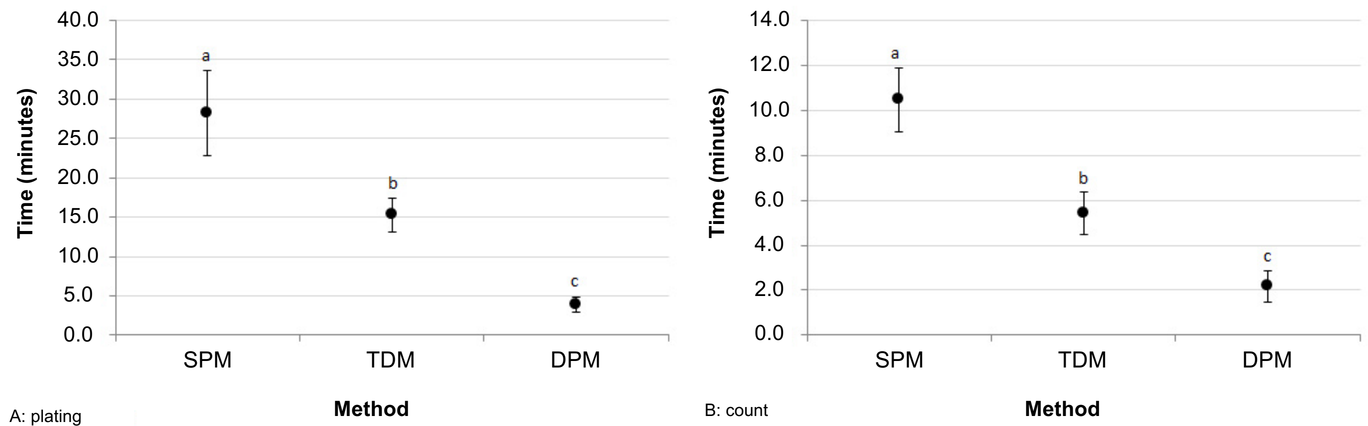
Table 2: Intraclass Correlation Coefficient values and respective 95% Confidence Interval for reproducibility analysis between the three different plating methods according to *Candida* species.

Species	ICC	$CI_{95\%}$		
Candida albicans	0.997	0.994	⊢⊣	0.998
Candida glabrata	0.994	0.989	⊢⊣	0.997
Candida krusei	0.993	0.983	⊢⊣	0.997
Candida tropicalis	0.946	0.899	⊢⊣	0.973

Caption: ICC: Intraclass Correlation Coefficient; $CI_{95\%}$: 95% Confidence Interval.

The results also showed that the two alternative methods were able to slightly shorten the worktime compared to the traditional method, concerning both plating and colony count (Figure 3).

Figure 3: Time mean (minutes), and standard deviation, for plating execution (A) and colony count (B) of *Candida* samples according to used method. Differences between means were considered statistically significant according to Welch's ANOVA (A: F=613,410; p<0.001/B: F=422,614; p<0.001). Different letters denote different means according to Games-Howell test (p<0.001).



DISCUSSION

Infections caused by *Candida* species are normally associated with biofilm formation by fungal species and are difficult to treat.[1] For this reason, studies have tried to comprehend the genetic and biochemical aspects of biofilm development and drug resistance as well as knowing the prevalence in vulnerable groups and establish new antimicrobial strategies.[31-32] In all these study types, it is fundamentally important to quantify in an accurate and reproductible way the number of viable cells in studied samples.

The estimate of the number of viable microorganisms in microbiological samples was described more than one hundred years ago and even today can be considered one of the pillars of microbiology. This quantification is typically performed by counting the total number of colony forming units (CFU) growth on a agar plate from serial dilutions. The conventional method used for CFU enumeration of *Candida* species is denominated spread plating (SPM).[11] However, SPM has drawbacks as the time spent for its execution and the large volume of necessary material.[12,14] Thus, alternative plating methods have been proposed in literature.

Referring to concordance between the evaluated methods in this study, it was observed that both alternative methods (Track-dilution method – TDM and Drop plate method – DPM) resulted, systematically, in $\log_{10}$(CFU/mL) values slightly higher in relation to traditional method (SPM). It has already been reported that SPM may underestimate CFU/mL values compared to alternative plating methods.[11] The usage of glass or plastic handle, necessary for sample spreading in SPM, may provoke injury to microbial cells and affect the number of CFU depending on spreading time and execution way, which could explain the current study results.

The difference between the means was considered statistically significant only for *Candida krusei* species, since the alternative methods were considered similar to each other. Thus, although the observed SPM tendency to underestimate the $\log_{10}$(CFU/mL) value, it seemed that this problem was in fact evidenced only for *C. krusei* species. The study leaded by

Walsh and colaborators[25] also showed the occurrence of weaker correlation between SPM and spiral technique for *C. krusei*, compared to results obtained with *Candida albicans* and *Candida glabrata*. *C. krusei* colony shape is different from other assessed species, they have a more widespread growth with rough texture and may require a shorter incubation time in order to avoid colony overlap and consequently count error. It was already suggested that, in single species cultures, the incubation time and dilution selected for count may depend on size of formed cells and colonies.[11] As the hypothesis of cell damage due to handle used in SPM was not assessed in *Candida* species, it cannot be said if SPM underestimated the colonies or, conversely, TDM and DPM overestimated the estimate. Although significant, *C. krusei* count difference observed in this study between SPM and other methods did not exceed, on average, 0.3 log, which may be a difference without practical importance, depending on required accuracy in different studies that come to use alternative methods. Thus, the *C. krusei* species plating with DPM and TDM should still be viewed with caution and could be investigated in the future using other strains such as, for example, clinical isolates.

Even so, the reproducibility analysis pointed out an adequate concordance between methods since all ICC values were above the value of 0.71, considered limit for a good concordance. *Candida tropicalis* was the species that presented the lowest ICC value and the most inaccurate $CI_{95\%}$. This result has already been suggested by Bland-Altman plot,[30] in which it is possible to verify greater dispersion of differences in log_{10}(CFU/mL) values between methods; some samples presented differences greater than 2 log. This result shows the importance of using more than one statistical method in analysis to conclude about the reliability between methods. Although ANOVA has not identified the differences in means for this species, the decision was close to the significance threshold which also draws attention to greater caution using alternative methods for this species.

The current study also sought to perform a comparison of procedure time of DPM, TDM and SPM methods for quantification of *Candida* species. The two alternative methods were able to slightly shorten the worktime compared to the traditional method. Shortening the time to perform plating was already expected because both methods (DPM and TDM) have very simplified stages in relation to SPM, and TDM procedure length in bacterial species has already been documented.[12] As regards the colony count, the most likely explanation for time reduction is related to the lower number of CFU obtained through alternative methods. Both in TDM and in DPM, the plating volume is 10 times smaller than SPM one, and, then, a smaller number of colonies is expected. For this reason, studies that use DPM indicate the colony count in dilution that presents between 3–30 colonies,[13] opposing to standard recommendation that is between 30–300 colonies.[13,18] Our results showed that the DPM execution, both colony plating and count, was faster than TDM and significantly faster than SPM. This result is directly related with the procedure of that method since it enables the use of multichannel pipette and plating of at least six aliquots in the same plate. Moreover, it was

already reported that the higher microorganism concentration in the sample, the greater the difference in time for colony count between DPM and SPM.[13] It is worth noting that, although the smaller volume and, consequently, lower number of formed colonies, the CFU value considered is always relative to the plating volume (CFU/mL or CFU/mg) without, in theory, discrepancies justified by these differences between TDM and DPM methods with SPM.

As main limitations of this study, the following is considered: the use of only one strain per species and the use of only one culture medium type for fungal species. Therefore, future studies could broaden the knowledge on this subject, investigating those other variables.

CONCLUSION

This study evidences, considering its limitations, make it possible to conclude that the null hypothesis that there is no effect from different plating methods in number of colonies recovered after plating can only be accepted for *Candida albicans*, *Candida glabrata* and *Candida tropicalis* species. Thus, these species can be plated through track-dilution method (TDM) and drop plate method (DPM) without damaging the quality of obtained data. Nevertheless, for *Candida krusei* species, some variations in colony count compared to spread plate method may be expected, and, then, the use of alternative methods should be done with caution depending on required objective and accuracy in each study. If the objective is not based on estimation of frequencies and prevalence in a certain population, and only on comparing the number of colonies between study groups instead, it is believed that the methods can be applied without damaging information. Relating to the execution time, the null hypothesis that there is no effect from different plating methods in method execution time was rejected. DPM and TDM considerably shortened the plating execution time and colony count of *Candida* species, and DPM is the one that requires the shortest time among the three methods.

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AVAILABILITY OF DATA

Does not apply.

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CONFLICTS OF INTEREST

There are no conflicts of interest to declare.

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