Intra-examiner Reproducibility of the Agar Plate Method for Estimation of Phospholipase Activity in *Candida albicans*

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Abstract

Although measurement errors can impair statistical analysis, reliability analysis has been neglected in applied microbiology. This study assessed the intra-rater reproducibility of the Agar-based method for estimation of phospholipase activity (Pz). Pz readings were performed twice by two examiners (E1, E2), either directly on plates or in photos, and both black and white backgrounds were used. Pz values were taken from one or triplicate colonies from each sample (n=30). Intra-examiner reproducibility was estimated using Intraclass Correlation Coefficient (ICC). For both examiners, reading triplicate (ICC_{E1}=0.91, ICC_{E2}=0.86) was better than one colony (ICC_{E1}=0.86, ICC_{E2}=0.80). E1 had an excellent concordance when measurements were performed on photos using a white background (ICC=0.95) and a good concordance in the other conditions (0.76<ICC<0.90). E2 showed good concordance irrespective of the conditions tested (0.74<ICC<0.89), but the use of a black background improved the ICC (0.80 to 0.89). Thus, a particular reading protocol may not be the best option for different examiners and experimental designs should attest intra-rater reproducibility prior definitive data collection.

Key Words: Intra-examiner reproducibility, Intraclass Correlation Coefficient, Phospholipase Activity, *Candida albicans*

1. Introduction

Oral candidiasis is a common superficial infection in humans that affects especially elderly denture wearers (Akpan & Morgan, 2002). Several *Candida* species have been frequently isolated from the oral cavity and dentures of patients with oral candidiasis, but *C. albicans* is the most virulent and pervasive of all, which is the reason for its pre-eminent position in the hierarchy of prevalence (Belazi et al., 2005; Darwazeh al., 1990; Dorocka-Bobkowska et al., 1996; Dorocka-Bobkowska et al., 2010, Gonçalves et al., 2006; Kadir et al., 2002; Manfredi et al., 2002; Motta-Silva et al., 2010; Pinto et al., 2008; Webb et al., 2005).

There are some local and systemic factors that are known to predispose oral candidiasis (Belazi et al., 2005; Darwazeh et al., 1990; Davenport 1970; Dorocka-Bobkowska et al., 2010; Farman e Nutt, 1976; Monroy et al., 2005; Ship et al., 2002). However, the infective ability of *C. albicans* also depends of several virulence factors. Among them, adhesion to host and abiotic surfaces, such epithelial cells and denture surfaces, as well as

biofilm formation, are considered the first step to initiate *Candida* infection (Chandra et al., 2001A; Chandra et al., 2001B; Ramage et al., 2004; Ramage et al., 2005). Additionally, the ability of *C. albicans* to produce hydrolytic enzymes, such as phospholipases (PLs) and secreted aspartyl proteinases (SAPs), is another important virulence factor (Calderone e Fonzi 2001; Lyon et al., 2006; Motta-Silva et al., 2010; Pinto et al., 2008). Such enzymes cause the rupture of the epithelial cell membrane and permit the penetration of the fungi cell into the host tissue (Hube 1996, Naglik et al., 1999, Banno et al., 1985, Samaranayake et al., 1984). Since these two enzymes play an important role in pathogenicity of *Candida* species, several investigations aimed to assess the presence of SAPs and LPs activity in *Candida* isolates (Price et al., 1982; Rüchel, 1999, Silva et al., 2012; Negri et al., 2010; Silva et al., 2011; Lyon et al., 2006; Pinto et al., 2008; Rajendran et al., 2010; Motta-Silva et al., 2010; Thiele et al., 2008; Tsang et al., 2007; Koga-Ito et al., 2006).

According to the literature, the synthesis of extracellular hydrolytic enzymes can be quantitatively evaluated in vitro by measuring the hyaline zones of precipitation around yeast colonies in some specific media (Price et al., 1982; Rüchel, 1999). The ratio between the diameter of the colony and the diameter of the precipitation zone gives an estimation of enzyme activity, which is designate as the Pz value (Silva et al., 2012; Negri et al., 2010; Silva et al., 2011; Lyon et al., 2006; Pinto et al., 2008; Rajendran et al., 2010; Motta-Silva et al., 2010; Thiele et al., 2008; Tsang et al., 2007; Koga-Ito et al., 2006). Although widely used in last years, authors frequently failure to describe how the measures were performed to obtain the Pz values. This is probably the most relevant factor concerning the comparison of results obtained among different published data. Some authors reported that they used a digital caliper (Thiele et al., 2008) or a computerized image analysis system (Tsang et al., 2007) to determine the diameter of the precipitation zone, but this information is omitted from most of the studies (Lyon et al., 2006; Pinto et al., 2008; Rajendran et al., 2010; Motta-Silva et al., 2010; Negri et al., 2010; Koga-Ito et al., 2006).

Reliability of the measures is another important information that are frequently missing from studies. It is well established that assessment of reliability permits to conclude about the amount of error inherent to the measurement, which will consequently determine the validity of the results (Kottner et al., 2011; Dunn, 2004; Mulsant et al., 2002). According to the Error Theory (Shoukri et al., 2004; Pasquali, 2009), every measure has a component of the actual value and another of error, the latter being random or systematic. Thus, researchers' training as well as the standardization of procedures and calibration are necessary steps to minimize the occurrence of errors (Campos, 2008). In this context, analysis of intra-rater reproducibility is an example of method that can provide information about quality of measurements and attest the reliability of results (Kottner, 2011). Despite its importance, this step has been neglected in studies of applied microbiology, which can compromise the reliability of the information obtained and hence the quality of studies.

Since measurement errors can seriously affect the statistical analysis and interpretation of data, it is imperative to evaluate its magnitude by calculating a coefficient of reliability (Shoukri et al. 2004, Kottner, 2011). Thus, the aim of this investigation was evaluate the intra-rater reproducibility of Pz measurers made in the Agar-base method for estimation of phospholipase activity in *C. albicans* cells. In addition, different protocols were tested and the intra-rater reproducibility for each one was calculated.

2. Materials and Methods

2.1 Experiment Design

This was a double-blind in vitro study. The outcome variable was the ratio of the diameter of the colony to the total diameter of colony plus zone of precipitation (Pz, Figure 1). Readings were performed by two independent examiners (E1 and E2), which were blinded in regard to sample to be evaluated.

Different protocols of reading were used according to three factors: i. number of replicates read: measurements were made in one colony or triplicate colonies from the same sample; ii. background: readings were performed against a black and a white (natural light) backgrounds; iii. reading device: measurements were taken directly on Petri dishes using a Digital Caliper (Mitutoyo Corporation, Kawasaki, Japan. Model CD-6 CS) and on digital images of plates using the Adobe Photoshop CS4®. Standard images were obtained with a digital professional camera fixed at 30 cm from the Petri dishes using a tripod.

Sample size determination followed the recommendations of Bland (2000) admitting α=5%, β=20%, a minimal expected concordance of 0.51 (Equation 1 and 2), plus 10% of lost samples (n=30). Intra-examiner reproducibility was estimated using Intraclass Correlation Coefficient (ICC) (Fisher, 1970); based on the value of ρ, the agreement level was classified according to criteria suggested by Fermanian (Fermanian, 1984) (Table 1). Statistical analyses were performed by one co-author which was blinded.

$$z' = 0.5 \ln\left(\frac{1+\rho}{1-\rho}\right) \quad (1) \qquad\qquad n = \left(\frac{z_{\alpha/2} - z_{1-\beta}}{z'}\right)^2 + 3 \quad (2)$$

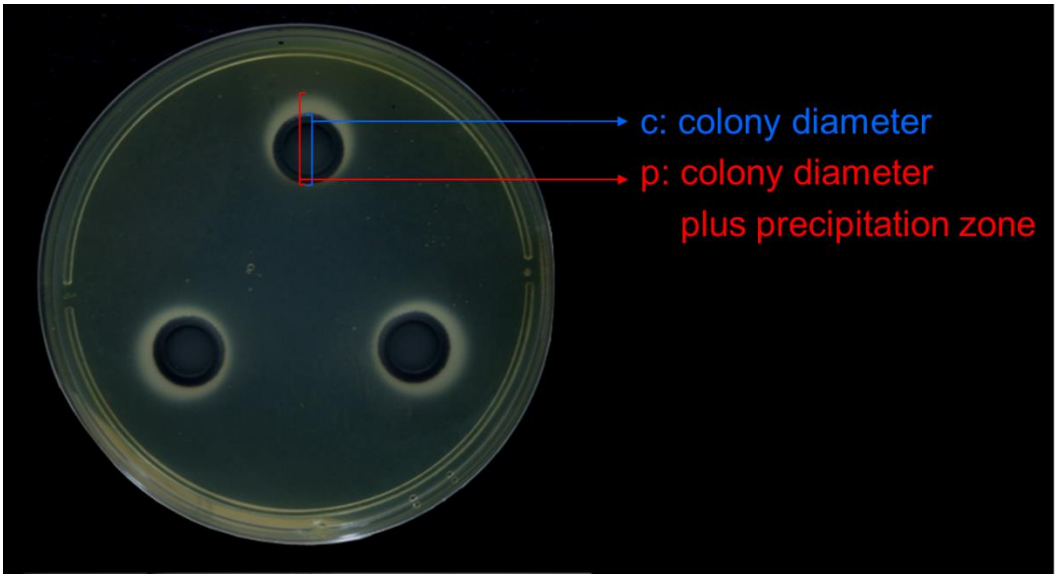

Figure 1: Estimation of phospholipase activity (Pz) according to Price et al. (1982). Pz was the ratio of the diameter of the colony to the total diameter of colony plus zone of precipitation

Table 1: Classification of agreement (ρ) according to Fermanian, 1984

ρ	Agreement
<0.31	Very Bad
0.31 ├ 0.51	Poor
0.51 ├ 0.71	Moderate
0.71 ├ 0.91	Good
≥ 0.91	Very Good

2.2 Microbiological Procedures

All procedures were performed by one independent examiner. This study used a reference strain of *C. albicans* from the American Type Culture Colection (ATCC 90028). The isolate was maintained in Yeast-Peptone-Glucose (YEPD) medium and frozen at -70°C until use.

The microorganism was subcultured onto Sabouraud Dextrose Agar (SDA - Acumedia Manufactures Inc., Baltimore, MD, USA) plates supplemented with chloramphenicol (0.05 g/l) and incubated at 37ºC for 48 h. To obtain the standard suspensions (n=30), a loopful of the agar stock cultures was transferred to 5 ml of Yeast Nitrogen Base broth (YNB - Difco, Becton Dickinson Sparks, MD, USA) supplemented with 100 mM glucose and incubated at 37ºC overnight in an orbital shaker at 120 rpm (Shaker, Quimis Aparelhos Científicos Ltda, Diadema, SP, Brasil). Cells of the resultant cultures were harvested and washed twice with phosphate-buffered saline (PBS, pH 7.2) at 5,000 x g for 7 minutes (Thein et al., 2007). Washed microorganisms were resuspended in sterile distilled water and spectrophotometrically standardized at an optical density at 520 nm (Biospectro, Equipar Ltda, Curitiba, PR, Brasil) to a final concentration of 10^8 cfu/ml (Negri et al., 2010).

Phospholipase production estimation was performed according to the methodology of Price et al. (1982) with a few modifications. A base medium was prepared using 10 g of peptone, 30 g of glucose, 57.3 g of sodium chloride, 0.55 g of calcium chloride, and 20 g of agar in 1 l of distilled water. The solution was autoclaved and, after cooling down to 50°C, the base medium was mixed to 80 ml of an egg yolk emulsion with potassium tellurite 0.15% (Laborclin Produtos para Laboratórios Ltda, Pinhais, PR, Brazil). Then, triplicate aliquots of 5 µl of cell suspensions (10^8 cfu/ml) were inoculated on the egg yolk medium and plates were incubated at 37ºC for 7 days (Negri et al., 2010). After incubation, the diameter of precipitation zones around the colonies and the diameter of the colonies were measured.

3. Results

The Intraclass Correlation Coefficient (ICC) based on analysis of variance (ANOVA) models has shown to be an adequate statistical method for reliability evaluations (Kottner et al., 2011). Firstly, we compute ICC for intra-rater reproducibility considering each factor (number of replicates read, background, and reading device), independently from the others (Table 2). In general, ICC values increased when Pz values were obtained from the mean of triplicate colonies, in comparison to reading only one colony per sample. However, only the ICC value of E1 changed its classification from ‘Good’ to ‘Very Good’, according to the criteria of Fermanian (1984). Considering the background color

and type of reading, no pattern was observed between examiners. While E1 had an improvement on its reproducibility using white background (from 'Good' to 'Very Good'), ICC value for E2 was superior using black background, but the classification of reproducibility was not altered. Performing readings in digital images improved the intra-rater reproducibility only for E1 (from 'Good' to 'Very Good').

Table 2: Intraclass Correlation Coefficient values obtained for intra-rater reproducibility

	Reading Colonies		Background		Type of Reading	
Examiner	Triplicate	One	Black	White	Caliper	Software
E1	0.91	0.86	0.86	0.93	0.84	0.94
E2	0.86	0.80	0.89	0.80	0.86	0.87

When the three factors were crossed, there were eight different protocols for each examiner (Table 3). A 'Very Good' reproducibility was achieved for E1 when readings were performed in triplicate colonies using digital images with a white background. All others protocols led to a 'Good' reliability for E1. The second examiner (E2) had their reproducibility classified as 'Good', irrespective of the protocol. However, it may be noted that the highest value of ICC for E2 was obtained for measurements performed in triplicate colonies on black background, regardless of reading with the Caliper or using the Software.

Table 3: Intraclass Correlation Coefficient values obtained for intra-rater reproducibility

		Caliper		Software	
Examiner	Colonies	Black*	White*	Black*	White*
E1	triplicate	0.76	0.90	0.90	0.95
	one	0.79	0.90	0.76	0.90
E2	triplicate	0.89	0.81	0.89	0.79
	one	0.83	0.85	0.77	0.74

* Type of background used to perform readings.

4. Conclusions

According to the results, all analyzed factors can lead to changes in the intra-rater reproducibility of Pz readings. For both examiners, reading triplicate colonies increased the ICC value. However, there was no pattern between the examiners when considering the other factors, suggesting that a particular reading protocol may not necessarily be the best option for different examiners. Although only two examiners were used in this study, it may be suggested that the experimental design of enzyme activity should include evaluation of the best reading protocol prior to definitive data collection. In addition, since independent examiners can have different performances on Pz readings, it seems important to evaluate inter-rater reproducibility in studies in which the measures will be carried out by more than one examiner

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