A modified crystal violet assay is comparable to XTT reduction assay for quantification of biofilm formation by *Candida albicans*

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Abstract

Background: The ability of the human fungal pathogen *Candida albicans* to form biofilms, for example on indwelling medical devices, is a major pathogenic mechanism and has been the focus of intense studies in the fungal pathogenesis field. A key research tool used is the quantitative methods for measuring biofilm formation of *C. albicans*.

Objective: We sought to optimize the conventional crystal violet (CV) staining assay for quantification of biofilm formation by *C. albicans* and evaluate its performance.

Methods: Individual modifications included (i) submerge-washing of microplates to remove non-adherent cells, (ii) heat-fixation, (iii) short-term staining for 3 min, (iv) submerge-washing to remove unbound CV dye, and (v) short-term destaining for 15 min were compared with the standard procedure, and those were superior were incorporated. Performance analysis was carried out for the modified CV assay, in comparison to the conventional CV assay and the XTT [2,3-bis(2-methoxy-4-nitro-5-sulfophenyl)-5-[(phenylamino)carbonyl]-2H-tetrazolium hydroxide] reduction assay.

Results: The modified CV assay demonstrated several advantages in quantitative assessment of biofilm formation of *C. albicans* over the conventional CV assay, including greater accuracy and reproducibility, shorter experimental time and reduced labor intensity, and was at least comparable to the XTT reduction assay.

Conclusion: The modified CV method can be used as an alternative to the XTT reduction assay in quantification of biofilm growth by *C. albicans*.

1. Introduction

Candida albicans is the most common fungal pathogen causing severe hospital-acquired infections in patients with implanted medical devices, and/or with depressed host immunity related to underlying diseases and immunosuppressive treatments [1]. Biofilm formation is an important virulence factor of *C. albicans*, as it provides a shield against antifungal treatments and human immune responses [1, 2]. Strains of *C. albicans* vary in their ability to form biofilms on standard biomaterials or biotic surfaces. This characteristic applies to *C. albicans* clinical isolates and genetically-manipulated mutants, and has been accepted as an indicator of strain virulence [3, 4]. Numerous assays have been developed to study biofilm formation of *C. albicans* [5]. Qualitative methods include scanning electron microscopy (SEM) and confocal laser scanning microscopy (CLSM) [4]. Results from qualitative assays are complemented by quantitative methods such as the colony forming unit (CFU) assay, the bioluminescence assay, the 2,3-bis(2-methoxy-4-nitro-5-sulfophenyl)-5-[(phenylamino) carbonyl]-2H-tetrazolium hydroxide (XTT) reduction assay, dry weight measurement assay, and the crystal violet (CV) assay [5, 6]. Each quantitative method has disadvantages, including not being amenable to high-throughput screening (CFU assay and dry weight measurement assay) [7], low-reproducibility (dry weight measurement assay and CV assay) [6,

8–10], unsatisfactory accuracy (XTT reduction assay) [11], long processing-time (CFU assay) [10], and high labor-intensity (CFU assay and ATP bioluminescence) [7].

Crystal violet assay for biofilm quantification was initially developed by Christensen *et al.* (1985), using 96-well microplates as the supporting substratum [12]. Its most widely accepted modifications include that by Deighton *et al.* (2001) [13] for *Staphylococcus epidermidis* and that by Otto *et al.* (1998) [14] for *Pseudomonas aeruginosa* and *Pseudomonas fluorescens*. Otto and colleagues (1998) used 95% ethanol as the eluting agent to extract crystal violet that bound to biofilms grown at the air-liquid interface and in the well bottom [14]. Their assay and its further modifications, including that by Jin *et al.* (2003) have been widely used in biofilm studies of *C. albicans*; however, its reliability and accuracy may be sub-optimal [8, 9]. This was likely due to the loss of adherent cells during washing and staining and the residue of unbound crystal violet after washing with pipettes [8].

The XTT reduction assay is a widely used quantitative method for biofilm formation of *C. albicans* [5]. The principle behind this assay is that tetrazolium XTT can be reduced to XTT formazan by the mitochondrial dehydrogenases of metabolically active yeast cells. XTT formazan is a water-soluble product that can be measured at OD₄₉₂. XTT reduction assay has been generally preferred to CV assays for quantification of *C. albicans* biofilms due to a better reproducibility; however, this assay suffers from problems such as unsatisfying accuracy and higher cost [11].

In this study, we introduced four modifications stemmed from Deighton's work (2001) into the conventional CV assay described by Gulati *et al.* (2018) [5, 13]. We show that the modified CV assay is superior to the conventional CV assay and is at least comparable to XTT reduction assay in quantification of biofilm formation by *C. albicans*, supported by higher accuracy, higher reproducibility and other merits.

2. Materials And Methods

2.1 *Candida albicans* strains

Five *C. albicans* strains that have been well-studied in regard to their biofilm characteristics were used (Table 1) [15–17], including a wild-type strain DAY286 and a mutant in the transcription factor Bcr1, which is a master regulator of biofilm formation by *C. albicans* [15, 17, 18], and a wild type strain CA14 and its mutants in the Als1 and Als3 cell wall adhesins. Biofilms formation of *C. albicans* strains lacking genes *bcr1*, *als1* and/or *als3* have been very well characterized [15, 16, 18, 19].

Table 1
Candida albicans strains used in this study.

Strains	Characteristics and sources	References
DAY286	Wild-type 1, biofilm-producer, from BWP17	[15]
<i>bcr1</i> ΔΔ	Biofilm-deficient mutant, from BWP17	[17]
CA14	Wild-type 2, biofilm-producer	[15]
<i>als1</i> ΔΔ/ <i>als3</i> ΔΔ	Biofilm-deficient mutant, from CA14	[16]
<i>als1</i> ΔΔ	Biofilm-deficient mutant, from CA14	[15]

2.2 Cultivation of biofilms in 96-well microplates

Biofilms were established in 96-well microplates (BD Falcon™, USA) as previously described [4]. Spider medium (1% nutrient broth, 1% D-mannitol, 2 g K₂HPO₄) was used as it supports the formation of typical *C. albicans* biofilms consisting of basal yeast layers and upper hyphal layers [4, 15]. *C. albicans* strains from – 80 °C stock cultures were streaked onto yeast peptone dextrose (YPD) agar plates (2% glucose, 2% peptone, 1% yeast extract, 2% agar) and incubated at 30 °C for 48 h. A single colony was transferred to 5 ml of YPD broth. After incubation at 30 °C for 18 h with shaking (200 rpm), cells were centrifuged at 2500 g for 5 min and washed twice with 1 ml PBS. The washed cells were then resuspended in Spider medium to an OD₆₀₀ = ~ 0.5 ± 0.05 (~ 1 × 10⁷ CFU/ml). This inoculum size was chosen as others revealed that 10⁷ CFU/ml of blastospores produced the most abundant and stable *C. albicans* biofilms [11]. 100 µl of the suspension was added to each microwell and the microplate was incubated at 37 °C with gentle shaking (75 rpm). After 90 min, the supernatant was removed and each microwell was washed twice with 100 µl of sterile PBS. 100 µl of fresh Spider medium was dispensed into each of the washed microwells, and the plates were further incubated at 37 °C with shaking at 75 rpm. The incubation was allowed for another 0 min (total time 90 min), 4.5 h (total time 6 h), 24 h, and 48 h, representing early adherence, early biofilm, intermediate biofilm and mature biofilm stages respectively [2, 20]. The medium was replenished after 24 h by aspiration of spent medium and adding fresh medium.

2.3 Quantification of biofilms

2.3.1 The conventional CV assay

The biofilm CV assay described by Gulati *et al.* (2018), referred to as the conventional CV assay in this study, was used as the basis for modifications as it has been widely used by others to study *Candida* biofilms [4, 5, 8, 21–24]. After biofilms were developed, cell suspensions were aspirated from the microplate using a multichannel pipette, and each microwell was washed twice with 200 µl of PBS to remove non-adherent cells. The washed biofilms were air-dried for 45 min at room temperature and stained with 110 µl of 0.4% CV for 45 min. After removal of unbound crystal violet, stained microwells were washed four times with 200 µl of PBS and immediately destained with 200 µl of 95% ethanol for 45 min. 100 µl of the solution was transferred to a new microplate and measured with a microplate reader (Infinite M200 Plate Reader, Tecan Group Ltd.) at 595 nm. Microwells containing growth medium

only served as the negative control. The absorbance values of the control wells were subtracted to minimize background interference.

2.3.2 Modification of the conventional CV assay

Five modifications from Deighton *et al.* (2001) were examined for their relative merits [13]. Only one modification was made each time. These modifications include 1) submerging the microplate into a container with distilled water three times to remove non-adherent cells, rather than washing the microplate with multichannel pipettes, 2) heat-fixing biofilms in microplates at 60–65 °C for 45 min, instead of drying biofilms at room temperature for 45 min, 3) staining the dried biofilms with Hucker's crystal violet (2%) for 3 min, instead of 0.4% crystal violet for 45 min; Hucker's crystal violet was selected because of its long-term stability and reliability in microbial staining [25], 4) submerging microplates in bulk distilled water four times to remove unbound crystal violet, rather than washing with a multichannel pipette, 5) eluting crystal violet from biofilms with 95% ethanol plus 5% acetic acid for 15 min at room temperature, rather than using 95% ethanol for 45 min. This change was supported by a previous study that found acetic acid was more effective than ethanol or ethanol-acetone (80:20) in releasing bound crystal violet dye, especially from thick biofilms [26]. After analyzing the merit of all five modifications respectively, a modified CV assay combining all advantageous modifications was developed.

2.3.3 XTT reduction assay

The XTT reduction assay was carried out as described by Gulati *et al.* (2018) [5]. After establishing biofilms in 96-well microplates and washing twice with PBS, 100 µl of XTT solution (containing XTT of 0.45 mg/ml, and phenazine methosulfate of 0.032 mg/ml) were immediately added into each microwell. The microplate was incubated in the dark for 30 min or 60 min at 37 °C. Incubation of *C. albicans* biofilms with XTT solution for longer time produces similar colorimetric changes [27]. The optical density was measured at 492 nm.

2.4 Silicone pad biofilm assay and fluorescence microscopy

Silicone pad biofilm assay and fluorescence microscopy were carried out as an independent and semi-quantitative reference assay to compare the accuracy of CV assays and the XTT reduction assay [20]. Sterile medical-grade silicone pads were pretreated with fetal bovine serum for 24 h at 37 °C, followed by washing with sterile PBS. Serum-coated silicone pads were immersed with a *C. albicans* suspension of OD₆₀₀ = 0.5 in a 24-well microplate for 90 min with shaking (75 rpm), washed with sterile PBS, and further incubated with fresh Spider medium at 37 °C with shaking (75 rpm) for 0 h, 4.5 h, 24 h, and 48 h. After rinsing with 1 ml of PBS, silicone pads were stained with 1 ml of calcofluor white solutions (0.05% [vol/vol]; Sigma) for 1 min, and then imaged with fluorescent microscopy at 405 nm [20]. The fluorescent intensity of each silicone pad was analyzed with Image J software (NIH, USA). The mean of nine individual values from three silicone pads was used to represent overall biofilm fluorescence intensities for an individual strain.

2.5 Statistical analysis

Assays were repeated on three occasions in quadruplicate (for microplate-based assays) or triplicate (for silicone pad-based assays). Means and standard errors were calculated. One-way ANOVA or the Mann-Whitney non-parametric test was employed to compare differences between the conventional CV assay and individual modifications, depending on data distributions (Minitab 16). The coefficients of variation (CoV) were calculated to assess the reproducibility of the assays. Differences in the overall CoVs of the XTT reduction assay, the modified CV assay, and the conventional CV assay for all four stages of biofilm formation, were examined by one-way ANOVA or the non-parametric test. Fluorescent microscopy was used as an independent reference to examine the accuracy of the CV assays and the XTT reduction assay. Agreement between the results obtained with these quantitative assays and the reference microscopy assay were assessed using Intraclass Correlation Analysis (ICA). As results from individual assays differ in units (CV: OD₅₉₅ readings; XTT: OD₄₉₂ readings; fluorescence microscopy: fluorescent intensity), ratios that represent differences in biofilm formation between wild-type and mutant strains were used for the evaluation of accuracy. $P < 0.05$ were considered statistically significant.

3. Results

3.1 Assessing individual modifications

3.1.1 Modification 1: Submerge-washing to remove non-adherent cells

In contrast to the conventional washing step using a multichannel pipette, submerging microplates in bulk distilled water generally led to lower readings of biofilms at different growth stages, with fewer early adherence monolayers (90 min) detected in all 3 mutant strains, and fewer mature biofilms (48 h) in both wild-type and 2/3 mutant strains (Fig. 1a and 1b). It appeared that submerge-washing removed not only non-adherent cells, but many loosely attached yeast or hyphal cells from *Candida* biofilms. Mutant strains of *C. albicans* often form structurally unstable biofilms with an outer layer composed of parallel rather than intertwined hyphae [15, 16, 18]. Thus, the conventional washing step with caution seemed to produce more accurate readings than submerge-washing of microplates, particularly for biofilm formed by *C. albicans* homozygous deletion mutants.

3.1.2 Modification 2: Heat-fixation

Although adding a heat-fixation step into the conventional CV assay did not cause any significant change in biomass of early adherent monolayers (90 min) (Fig. 1a), most mature biofilms (48 h) showed greater biomass [(148 ± 37) %, $p < 0.001$] (Fig. 1b). This suggested that adding heat-fixation resulted in less loss of adherent cells in the subsequent staining and washing steps.

3.1.3 Modification 3: 3 min CV Staining

In contrast to the conventional CV assay, which employs 45 min staining with 0.4% CV, staining with Hucker's CV for 3 min showed no significant difference in biofilm readings (Fig. 1a and 1b). While being equivalent to the longer staining time, this modification reduced the experimental time by 42 min. As noticed in our study, as well as others' [11], it was important to add 10% extra volume of the staining

solution relative to that of the initial *Candida* suspension. This ensured that biofilms grown at the air-liquid interface and in the bottom of the microwell were both sampled.

3.1.4 Modification 4: submerge-washing to remove unbound crystal violet

In general, no significant difference in biofilm readings was found when unbound crystal violet was removed by either submerge-washing of the microplate or washing with a multichannel pipette (Fig. 1a and 1b). Submerge-washing, however, was less labor-intensive than the conventional washing procedure and more reproducible when examining mature biofilms, as reflected by its lower CoV (0.31 ± 0.15 vs 0.48 ± 0.07 , $P = 0.04$). No difference in reproducibility was found when early adherence of *C. albicans* were examined ($P = 0.61$).

3.1.5 Modification 5: Destaining for 15 min

Destaining monolayers or biofilms with 95% ethanol and 5% acetic acid, showed similar results to those obtained with the conventional method (Fig. 1a and 1b). Modifying this step shortened the experimental time by 30 min.

All modifications excepted the first submerge-washing to remove non-adherent cells were incorporated into the conventional CV assay. Detailed protocol of the modified CV assay can be found in the supplementary material.

3.2 Comparing the modified CV assay, the conventional CV assay and the XTT reduction assay

C. albicans biofilms grown to different stages were quantitatively assessed by the modified CV assay, the conventional CV assay, and the XTT reduction assay. Performance of each assay was analyzed and compared.

3.2.1 Reproducibility

The coefficient of variation (CoV) was used as the indicator for the reproducibility of each assay. When the entire biofilm formation processes were examined on three different occasions in quadruplicate, the XTT reduction assays showed a significantly lower CoV than the conventional CV method (0.19 ± 0.12 vs 0.36 ± 0.22 , $p = 0.006$), suggesting higher reproducibility. The modified CV method also had a significantly lower CoV than the conventional CV method (0.25 ± 0.14 vs 0.34 ± 0.21 , $p = 0.04$). No difference in reproducibility was found between the XTT reduction assay and the modified CV assay ($p = 0.13$).

3.2.2 Accuracy

The fluorescence microscopy assay was carried out as an independent reference, using same strains and medical-grade silicone pads. Gross morphology and fluorescence densities of *C. albicans* biofilms of different growth stages were presented in Fig. 3a and 3b. The quantitative changes of biofilms over the time course demonstrated by the XTT reduction assay were noticeably different from that demonstrated by qualitative and semi-quantitative microscopic analysis (Fig. 2c and Fig. 3). Biofilm readings from CV assays were in a better agreement. To validate this, we determined the intraclass correlation coefficients (ICC) between each of the three quantitative assays (the modified CV assay, the conventional CV assay

and the XTT reduction assay) and the results of microscopy. To avoid the effect of the difference in units, for comparison, we used the relative difference (ratios) in biofilm formation between the wild-type and mutant strains. Throughout all stages of biofilm formation, the XTT reduction assay showed a low ICC (0.139) with the microscopy analysis, while the conventional CV assay and the modified CV assay had a moderate correlation (0.469) ($p < 0.001$) and a higher correlation (0.546) ($p < 0.001$) respectively. This suggests that the modified CV assay has the highest agreement with the independent semi-quantitative microscopic assay when assessing mature biofilms, followed by the conventional CV assay and the XTT reduction assay.

3.2.3 Other considerations

Other factors, such as the time used to complete the assay, labor intensity and cost have also been compared. The modified CV assay and the XTT reduction assay demonstrated advantages over the conventional CV assay (Table 2). Both XTT reduction assay and modified CV assay required much less time (~ 2 h) than the conventional CV assay. Omitting excessive washing by XTT reduction assay or replacing the conventional mechanical washing step with submerge-washing by modified CV assay significantly reduced labor.

Table 2
Strengths and weaknesses of three biofilm quantitative assays.

	Modified CV assay	Conventional CV assay	XTT reduction assay
Time to complete	~ 1.5-2 h	~ 3.5-4 h	~ 1.25– 1.75 h
Cost	Low	Low	Relatively high
Labor intensity	Intermediate	High	Low
Reproducibility	High	Low	High
Accuracy*	High	Intermediate	Low to intermediate
*Accuracy was assessed using semi-quantitative fluorescence intensity of biofilms grown on silicone pads as references. Ratios that represent differences in biofilm formation between wild-type and mutant strains were used for the evaluation of accuracy.			

3. Discussion

Here we sought to optimize an *in vitro* biofilm quantitative assay for *C. albicans*, taking into consideration high-throughput potential, time required to complete the assay, labor-intensity, and more importantly, reproducibility and accuracy. The 96-well microplate is an ideal *in vitro* biofilm cultivation and quantification platform for high-throughput screening [28]. The presence of 96 microwells in one plate allows rapid processing of a large number of fungal strains. Identical roughness and surface free energy of each microwell yield comparable and controllable biofilms. Although the plate material, polystyrene, is not medically relevant, wide commercial availability, sound knowledge of polystyrene-grown biofilms, and

excellent ability to promote cell adherence all make it a material of choice for many diagnostic and research laboratories [29].

The modified CV assay, described herein incorporated advantageous features of methods described by Deighton *et al.* (2001) and O'Toole *et al.* (1998) [13, 14]. Modifications incorporated into the conventional CV assay showed improvements in several aspects. Heat fixation improved retention of *C. albicans* biofilm biomass during washing and staining and resulted in a more accurate estimation. *C. albicans* biofilms consist of filaments originating from the basal layer of yeast cells and such filaments can readily slough off the biofilms [15]. Occasionally, the entire biofilm in a microwell might be lost during the aspiration or washing step [30]. Such a partial or entire loss of *C. albicans* biofilms in microplates before quantitation significantly affects the results. It has been reported that air drying, even when combined with methanol was inadequate for fixing *Candida* biofilms to the substratum [9]. Heat-fixation increased the stability of candida biofilms on the substratum against external forces such as vigorous washing [15, 20, 31]. Washing with bulk distilled water to remove the unbound CV stain is associated with higher reproducibility, as supported by a lower CoV, and is much less labor-intensive. Deighton *et al.* (2001) found that using a manual pipette or mechanical plate washer for plate washing might disrupt biofilms unless extreme care was taken [13]. A volume of washing liquid greater than the well volume was also necessary for complete removal of any unbound CV residue on the sides and fringes of the wells, if pipette washing was adopted. The unsatisfactory reproducibility of conventional CV assay observed in our study was also in agreement with studies published by others [8, 21]. In contrast, the submerge-washing procedure has the ease in applying uniform treatment to individual microwells and thus improved the reproducibility [13, 32]. Short-term staining and destaining make the modified CV assay less time-consuming while still retaining the efficacy in detecting biofilm biomass.

The accuracy of the modified CV assay was found to be even higher than that of the XTT reduction assay, when fluorescent microscopy was chosen as an independent reference. CFU-based viable count assay is often considered as the gold standard that accurately assess biofilm formation of *C. albicans*. We used semi-quantitative fluorescent microscopy of biofilms grown on silicone pads as it also vigorously reflects biofilm growth in a microplate [33, 34]. Results from our modified CV assay compares well with other reports on biofilm formation of *bcr1ΔΔ*, *als1ΔΔ*, and *als1ΔΔ/als3ΔΔ* mutant strains [15–17, 19]. The XTT reduction assay, as found in this study and also by others [11, 21, 24], failed to detect differences in biofilm formation between some wild-type and mutant strains. This can be reasoned that while the modified CV assay detects all cells throughout the biofilm, XTT reduction assay only reflects the respiratory activities of cells residing in the blastospore layer of the biofilm [11, 35].

Conclusion

This modified CV assay is superior to the conventional CV assay and is at least comparable to XTT reduction assay for quantification of biofilm formation by *C. albicans* in terms of reproducibility, accuracy, experimental time and lab-intensity. Application of this modified method could also be extended to other fungi and bacteria.

Declarations

ETHICS APPROVAL AND CONSENT TO PARTICIPATE

Not applicable.

CONSENT FOR PUBLICATION

Not applicable.

AVAILABILITY OF DATA AND MATERIALS

On request.

CONFLICT OF INTEREST

The authors declare no competing interests.

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AUTHORS' CONTRIBUTIONS

YQ and FS designed the experiments. SY and YQ performed the experiments. YQ, FS, and ZC analyzed the data, YQ and FS prepared the manuscript. All authors have read and agreed the final manuscript.

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Figures

Figure 1 A)

B)

Figure 1

Biofilm formation of *C. albicans* determined by the conventional crystal violet (CV) assay and five modifications. *C. albicans* biofilms were grown in 96-well microplates for 90 min (Fig. 1a) or 48 h (Fig. 1b) and quantitatively determined by the conventional CV assay and 5 modified versions of this assay with a single modification incorporated, as described in the text and indicated in the Figure. Five *C. albicans* strains were examined, including *C. albicans* DAY286 [wild type (WT) 1] and its *bcr1* homozygous deletion mutant, and CA14 (WT 2) and its *als1* and *als1/als3* homozygous deletion mutants. The readings for the mutant strains were normalized to that of the corresponding wild type strains to simplify the analysis. Experiments were repeated three times in quadruplicate. Error bars represent the standard error of the mean. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$.

Figure 2

Figure 2

Assessing *C. albicans* biofilm formation using different quantitative assays. *C. albicans* biofilms were grown in microplates for 90 min, 6 h, 24 h and 48 h and quantitatively determined by the conventional crystal violet (CV) assay (Fig. 2a), the modified CV assay (Fig. 2b) or the XTT reduction assay (Fig. 2c). Experiments were repeated three times in quadruplicate. Shown are the means and the standard errors of the mean.

Figure 3 A)

B)

Figure 3

Fluorescence microscopy analysis of *C. albicans* biofilms. Biofilms of the indicated *C. albicans* strains were grown on serum-coated medical-grade silicone pads. Biofilms grown to different stages were washed, stained with calcofluor white solutions (0.05% [vol/vol]) for 1 min, and imaged with fluorescent microscopy at 405 nm (Fig. 3a). Fluorescent intensity of biofilms was analyzed by Image J, representing

the biomass of biofilms formed by different strains at different stages (Fig. 3b). Experiments were repeated three times. Error bars represent the standard error of the mean.

Figure 4

Differences in biofilm formation between wild type *C. albicans* and biofilm-deficient mutants assessed by different quantitative assays. Biofilms were grown in microplates for 90 min, 6 h, 24 h and 48 h. Differences in biofilm formation between *C. albicans* WTs and their biofilm-deficient mutants were

assessed with the conventional CV assay (Fig. 4a), the XTT reduction assay (Fig. 4b), the modified CV assay (Fig. 4c), and fluorescence microscopy (Fig. 4d) (also see Figures. 2 and 3). Experiments were repeated three times in quadruplicate (XTT and CV assays) or triplicate (fluorescence microscopy). Error bars represent the standard error of the mean.