

Comparative study for identification of *Candida albicans* with germ tube test in human serum and plasma

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Abstract

The aim of the study was to compare the technique of germ tube in human serum and plasma for the identification of *Candida albicans*. A cross-sectional prospective study was conducted at La Fe Clinic and the institute "Instituto Nacional de Salud del Niño". Prior informed consent, we obtained plasma samples (voluntary donation) and serum (vein removal) of an apparently healthy donor, without consumption of antifungals, respecting the norms of institutions, quality standards and the inclusion criteria. The samples were stored in cryovials to $\leq 20^{\circ}\text{C}$ until processing; germ tube technique was performed under a conventional method. *C. albicans* ATCC 14053 was used as a control. A total of 150 tests germ tube technique in plasma and 150 trials in human serum were included for the identification of *C. albicans*. The sensitivity, specificity, positive predictive value, and negative predictive value were 100%, 99.3%, 99.3%, 100 %, respectively ($\kappa = .99$, CI 95%: range: 96–1.01). This study allowed us to demonstrate that the technique of germ tube in human plasma is comparable with the technique of germ tube in human serum for the identification of *C. albicans* showing high performance and diagnostic agreement.

Introduction

The progressive increase in incidence of fungal infection over the last three decades is of great importance for communities worldwide and it has as its principal agent the genus *Candida*, mainly *Candida albicans* (*C. albicans*), amply isolated species and prevalent in clinical samples. *C. albicans* produces mucocutaneous infections, as an opportunist pathogen in oral candidiasis and vulvovaginitis, and exceptionally causes systemic invasive candidemia, principally in critical and immunocompromised patients with AIDS, autoimmune diseases, and transplant recipients [1-4]. The invasive sepsis causes high morbidity and mortality, high hospitalization costs and stays which represent almost a third of nosocomial infections in ICU [5,6]. The invasive mycosis is considered the second most frequently nosocomial infection in Peru, and the fourth in the United States, hence, must perform precise and rapid mycology diagnosis and, that allow corroborating the clinical diagnosis and the establishment of an adequate antifungal treatment.

The assays for phenotypical identification and differentiation of *Candida* species are automatized, commercial and conventional methods. The first methods are miniaturized biochemical assays of fermentation and carbohydrates assimilation. The commercial methods, such as CHROMagar, offer a rapid identification, diagnosis confection and recognition of organism mixtures with similar characteristics, but these presents' high costs, disabling its routine use in clinical laboratory, orienting the cost-benefit as a limitation. The yeast development on Sabouraud Dextrose Agar 2% (gold standard), the zymogram-auxogram and tube germ test are classically used methods. Although these tests present critical points, they are the most used altogether.

From the first research of Reynold and Braude [7], Livingood and Johnson [8], Taschdjian C, et al. [9], and Mackenzie [10] to those made by Kaminsky and Quinlan [11], Berardinelli and Opheim [12],

and Hilmioğlu et al. [13], the germ tube test is widely considered the better method for the identification of *Candida*. The germ tube is most economical, rapid, easy and used test for discriminating *C. albicans* and *C. dubliniensis* from other species, up to a 98% of sensitivity [12-14]. Nevertheless, this test form part of the three screening tests for differentiation of *C. albicans* to other species.

This test is realized in a diverse range of media, such as water agar with 1% milk, BHI, commercial media (broth SST, TSA-BAP, BHI, YEPD free-serum, among others), human serum (fresh, inactivated or frozen) being the most appropriate and feasible for filamentation [10,11,15]. Nowadays some modifications of the germ tube test have been developed, being most of the times low-cost-benefit and weakly applicable in screenings at health-care centers, principally in the middle-and-low-income countries.

The use of human plasma as an alternative is a possible additional modality of the test. Most health centers can obtain human plasma, for example, from aliquots of the separation of blood components in blood banking services. Likewise, plasma can be derived from hematology or biochemical areas in medical laboratories. In developing countries, with limitations in health services and prevalent diseases, the mycological diagnosis of Candidiasis requires a high efficiency, high sensibility, and relatively low costs, because new applications and/or modifications of diagnostic methods are required.

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We aimed to compare the technique of germ tube in human plasma and serum for the identification of *Candida albicans*.

Materials and methods

Clinical characteristics and donation of plasma and serum

A non-experimental, cross-sectional study was designed and developed at Instituto Nacional de Salud del Niño (INSN) and the Primary-health Center *La Fe* Clinic both in Lima, Peru. The study had the approval of the Committee of Ethics and Research from the Research Support Department of the institution. The sample size was calculated using EPIDAT v.4.1 (Xunta de Galicia, Spain) considering a sensitivity of 0.95, heterogeneity of 50% and an accuracy of 0.04 obtaining a minimum sample size of 120 assays of the germ tube.

Having an informed consent, the samples of an asymptomatic and apparently healthy, donors in the phlebotomy and hematology area in *La Fe* Clinic, following the next exclusion criteria: a) Serum sampling: blood sample must have a minimum volume of 5 ml; lipemic, hemolyzed or evidently contaminated samples; samples were processed for two hours and refrigerated or stored at $\geq 4^{\circ}\text{C}$. b) Plasma sampling: incomplete blood donation or with complications, and no stored fresh plasma or stored at $\geq 70^{\circ}\text{C}$.

The serum sampling was realized with BD-Vacutainer® system (Franklin Lakes, New Jersey), following the CLSI H26-A3 y H03-A6 guidelines [16,17]. Each sampling was obtained around in seven tubes, and an additional tube with EDTA or heparin was included for CBC (Complete blood count) and biochemical parameters, as glucose, total proteins, cholesterol, etc. (Figure S1 – supplementary appendix) [10,18]. The plasma sampling was performed in the blood banking service of Hospital Nacional Docente Madre Niño San Bartolomé, to

Lima, Perú, which met the requirements of the manual of technical competence and normalized proceedings referred to the institution [19]. Aliquots derived from fresh plasma were fractionated and stored in 2 ml cryovials (Samplix®, Laboratorio Muestreo y Análisis, Peru) at $\leq 70^{\circ}\text{C}$ (Figure 1) [20].

Pre-analytical phase

During the second trimester of 2015, eight strains of *C. albicans* and *C. non-albicans* (*C. glabrata*) were selected and collected from paediatric (children) patients and identified with Vitek® 2 Compact (BioMérieux, Marcy-l'Étoile, France) in the microbiological service of INSN. We used *C. albicans* ATCC 14053 (American Type Culture Collection, Manassas, US) as a performance control in the germ tube test. The cultures were stored in the mycology library to INSN, they were reactivated and isolated in sabouraud dextrose agar (Merck, Darmstadt, Germany) for germ tube assessments, where they were incubated at $36 \pm 1^{\circ}\text{C}$ for 18 to 24 hours [20].

Analytic phase

The culture of *C. albicans* strains and ATCC (positive control) was performed in the INSN. A colony was lightly touched with a straight wire and then inoculated in the test tubes containing human plasma and serum. The test tubes were incubated for 2 hours at 37°C . For reading the test two drops of the aliquot from each test cryovial (human plasma and serum) was withdraw whit a loop, placed on a slide (before submerged in alcohol to avoid fungal growth) and evaluated under a magnification of 40x and 100x for the presence/absence of germ tube.

We considered as a real and positive germ tube structure the elongated daughter cells originated from the round mother cell without any constriction at their origin, and the constriction of the hyphae at

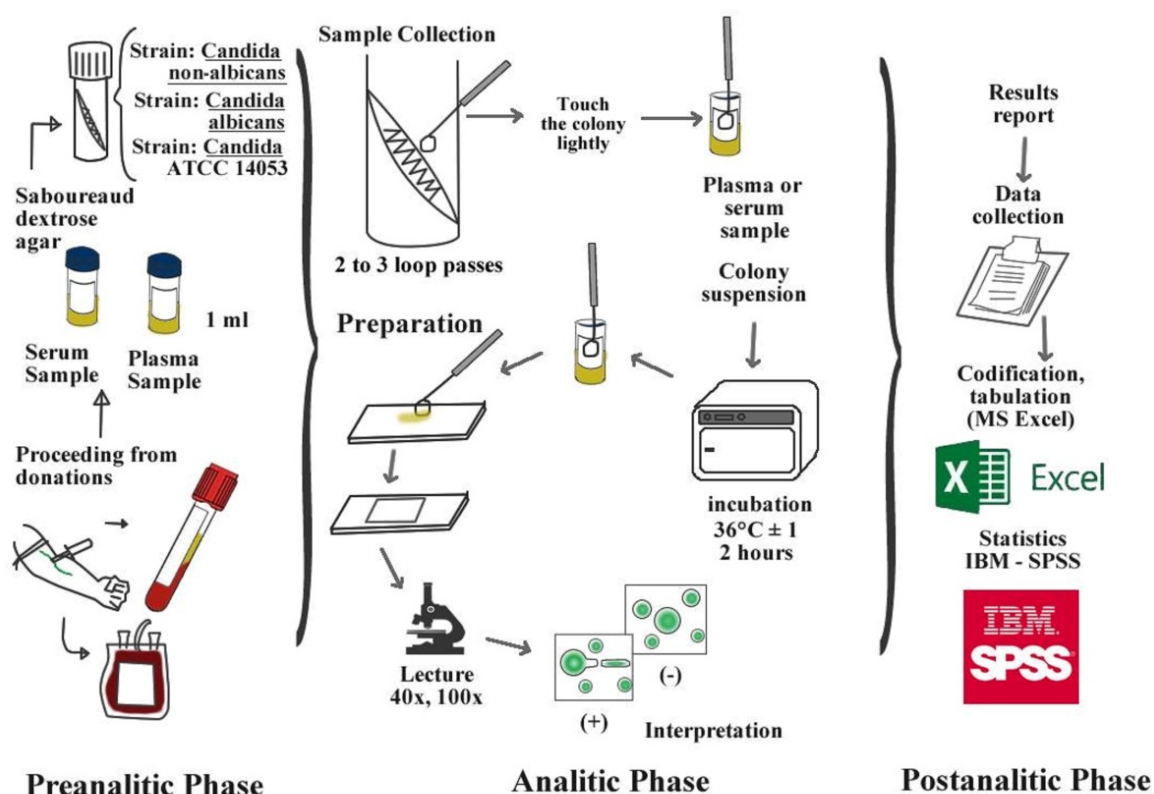


Figure 1. Flowchart of blood extraction, pre-processing and processing germ tube test in human plasma and serum (colour online only)

the round mother cell was referred to as pseudo hyphae or sprout of itself [7,10,21]. The germ tube has half the width and three to four times the length of a yeast cell. A minimum of seven and twelve fields from the germ tubes was observed respectively as a criterion for germ tube test positivity and negatively.

Data analysis

Statistical analysis was performed with IBM SPSS v20 (Armonk, USA) for Windows. The diagnosis test was used for comparing the sensitivity of germ tube test in human plasma and serum, considering as a reference standard the germ tube test in human serum. The overall Cohen's Kappa (κ) inter-test was used and Coeficiente Interval of 95% was considered statistically significant.

Results

We included 300 assays (150 for each type of media) that are shown in Table 1, where all false positives are also described. We performed 250 assays with *C. albicans* and 50 assays with *C. non-albicans* (*C. glabrata*), all the assays were validated with simultaneous processing of *C. albicans* ATCC 14053.

Table 2 show the diagnostic test, where all positive germ tube tests in human serum also presented filamentation in human plasma. The yield diagnosis of the test in human serum was 83.5% (150 positive tests) statistically comparable with the values for human plasma 83% (149 positive assays) ($p = 0.120$). Neither method presented false positive results, κ test indicated an excellent agreement between germ tube in plasma and serum ($\kappa = 0.99$, IC 95%: 0.96 to 1.01) (Table 1,2).

Discussion

In this study, we scrutinized the germ tube test in human plasma compared with human serum as an important tool for the identification of *Candida* species, principally *C. albicans*, showed an excellent agreement and yield diagnosis. Also, iterate its possible cost-benefit aspect.

The rapid identifications of *C. albicans* are crucial and important in the clinical laboratory because it represents an independent risk factor for invasive candidiasis, it is the most important cause of mortality in critical patients and it is a significant problem of Global Public Health [5,15,22]. Numerous scientific evidences suggest that the early

implementation of the adequate antifungal therapy is a critical factor in improving the outcome of systemic invasive mycosis occasioned by *C. albicans*, principally in pediatrics and immunocompromised patients [23-25]. It is noteworthy that the opportune management of these cases correspond to a correct and early diagnosis, where the germ tube test constitutes the initial and discriminatory measure for identification of *C. albicans*. Although it is not the definitive diagnostic test, it is discriminatory for other complementary tests in the correct scheme and it is the most used method in clinical laboratories worldwide [7,12,14].

Otherwise, we show a low rate of false negatives (0.4%) and that all negative samples in human serum were negative in human plasma (Table 2). The interpretation of the test as positive is indicated by the production of germ tubes: the growth of filamentous mycelium (morphological transition representing a response to changing environmental conditions). Positive results for germ tube are indicative, preliminary, of the identification of *C. albicans* and/or *C. dubliniensis*, which can be confirmed and corroborated with different biochemical, molecular and morphological evidence [13,26,27]. The results of this study showed that there exists a correlation in the germ tube test in plasma compared with human sera (Table 1) of strains from cultures identified morphologically and biochemically; results that can be extrapolated to direct determinations from clinical samples, as demonstrated previously in different studies [21,25,28].

The human plasma is an ideal medium for mycotic filamentation of *C. albicans* which has 100% of specificity, 99.6% of sensitivity and 99.7% of accuracy (Table 2). These results are comparable with other studies for validation of commercial media, where it is reported a ~96% of sensitivity and ~98% of especificity in several media [7,10,26,27,29-31]. The agreement level between human plasma and serum was excellent ($\kappa = 0.99$), given that values lower than 0.39 indicate a poor agreement, the values between 0.40 and 0.59 indicate a moderate agreement, and results higher than 0.60 show a substantial and almost perfect agreement. Our results showed an excellent agreement in the determinations of germ tube test for *C. albicans* [32]. The agreement observed was 1.00 and the agreement that can be expected from random chance was 0.72 (Table 1).

Deorukhkar et al. [31] demonstrated that the human plasma is the third best media for the germ tube test in the identification of *C. albicans* y *C. dubliniensis* and Berardinelli y Opheim [12] showed an optimal yield with plasma of rabbit. For both, the sobreinoculum generated opposite results that reduced the yield method. Moreover, these methods used trypticase-soy broth (like others who use culture media and rabbit substrates), being these results low cost-benefit and more laborious since for its use as a routine diagnostic method, it should be considered the difficult organization of the sanitary centers and the massive concurrence of patients for diagnosis and treatment.

During the period of the study, there were no studies in Peru that compared human plasma and serum as a media for the development of germ tube in the identification of *C. albicans*. Our results demonstrated a potential utility of human plasma in germ tube testing. Human plasma could be obtained from aliquots derived from the Blood Bank Service, where it secures the media free more or less from pathogens [Human Immunodeficiency Virus (HIV), Human T-lymphotropic virus types-1 and -2 (HTLV), hepatitis B virus (HBV), syphilis, etc.].

In summary, this study allowed us to demonstrate that the technique of germ tube in human plasma compares to the technique of germ tube in human serum for the identification of *C. albicans* showing

Table 1. Shows an agreement between human plasma and human serum (n=300)

SGT	PGT		
	Positive	Negative	Total
Positive	149	1	150
Negative	0	150	150
Negative	149	151	300
Total	0.99	CI 95%	
		0.96 a 1.01	

SGT: Serum Germ tube Test; PGT: Plasma Germ tube Test

Table 2. Shows a diagnostic test for germ tube in human plasma

Components		CI 95%
Sensitivity	99.6%	97.8% a 99%
Specificity	100%	92.9% a 100%
Valor Predictivo Positivo	100%	98.5% a 100%
Valor Predictivo Negativo	98%	89.7% a 99.7%
Proportion of False Positives	0%	0.0% a 0.71%
Proportion of False Negatives	0.4%	0.1% a 2.2%
Accuracy	99.7%	98.1% a 99.9%

a high performance and a diagnostic agreement.

Author contributions

JMS and RRL conceived and designed the experiments. JMS and RRL performed the experiments. JMS analyzed the data and wrote the paper.

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Conflict of interest

The authors declare that there are no conflicts of interest.

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