Diagnostic Microbiology and Infectious Disease 93 (2019) 39–43

Contents lists available at ScienceDirect

Diagnostic Microbiology and Infectious Disease

journal homepage: www.elsevier.com/locate/diagmicrobio

Mycology

Performance of *Candida albicans* germ tube antibodies (CAGTA) and its association with (1 → 3)-β-D-glucan (BDG) for diagnosis of invasive candidiasis (IC)

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ARTICLE INFO

Article history:
Received 21 May 2018
Received in revised form 5 July 2018
Accepted 6 July 2018
Available online 8 August 2018

Keywords:
Candida albicans germ tube antibodies
CAGTA
(1→3)-β-D-glucan
Invasive candidiasis
IC

ABSTRACT

Invasive candidiasis (IC) plays an important role as severe infection. Elder population, immunocompromised individuals, and intensive care unit (ICU) patients, especially when exposed to major surgery, are the most affected. IC diagnosis and treatment are difficult because of the absence of pathognomonic signs and symptoms. In addition, culture-based examination (gold standard) is known to have low sensitivity and long time to report. All these often lead to unnecessary and costly empirical antifungal therapies, burdened also by the onset of drug resistance and serious side effects for the patient. To partially overcome these problems, in recent years, novel noncultural markers have been investigated with the aim of easily and rapidly achieving an early diagnosis of IC. Such novel markers include the pan-fungal antigen (1 → 3)-β-D-glucan (BDG) and the anti–*Candida albicans* germ tube antibodies (CAGTAs). We retrospectively analyzed the presence of CAGTA on −80 °C stored serum samples, where the level of BDG had been previously assessed in a prospective study conducted in the Azienda Ospedaliero–Universitaria Policlinic of Modena (Pini et al. Infection 44:223–233, 2016). In particular, we selected 29 samples from proven IC episodes and 28 from non-IC cases. The 29 IC samples had been diagnosed as infections by *C. albicans* ($n = 16$), *C. glabrata* ($n = 8$), *C. parapsilosis* ($n = 1$), *C. pelliculosa* ($n = 1$), and *C. tropicalis* ($n = 1$), while 2 samples had intrasurgery biopsies positive for yeast (compatible with *Candida* spp.). The 28 control samples (non-IC) included 9 sera with positive blood cultures [*E. faecium* ($n = 5$), *S. pneumoniae* ($n = 2$), *P. aeruginosa* + *A. baumannii* ($n = 2$)] and 19 negative blood cultures. The CAGTA immunofluorescence assay was performed using 1:40, 1:80, 1:160, and 1:320 dilutions (reference dilution, as indicated by the manufacturer). According to the protocol, the samples were evaluated by the operator-dependent optical reading based on immunofluorescence positive/negative samples. In parallel, with the aim of standardizing the reading, the fluorescence images were captured, and the data were expressed as arbitrary fluorescence units (AFU). Finally, the results were interpreted as positive or negative using a cutoff provided by receiver operating characteristic (ROC) curves (Youden index). The traditional operator-dependent optical reading and the AFU measuring protocol provided comparable information with respect to the processed samples since IC and non-IC sera were correctly identified by the 2 CAGTA reading strategies in most of the cases. Interestingly, the AFU reading enabled a semiquantitative evaluation of the samples and an objective interpretation of the results. Based on the cutoff value, the AFU-based CAGTA procedure demonstrated a sensitivity of 52% and a specificity of 89%, while BDG showed a sensitivity of 90% and a specificity of 75%; the overall accuracy was 70% and 83% for CAGTA and BDG, respectively. The association of the 2 markers greatly increased both sensitivity and accuracy to 97% and 84%, respectively. As expected, when excluding non–*C. albicans* episodes, the sensitivity of CAGTA increased from 52% to 86%; moreover, with the exclusion of the non–deep-seated episodes, the sensitivity of CAGTA increased to 67% and reached 100% for *C. albicans* deep-seated candidiasis. Finally, when evaluating the influence of colonization, BDG demonstrated the most drastic decrease in specificity that dropped from 88% in noncolonized to 58% in colonized patients. With the exception of non–*C. albicans* episodes, CAGTA is a good marker of IC, particularly in the presence of deep-seated candidiasis. The performance of CAGTA greatly increases when used in combination with BDG.

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https://doi.org/10.1016/j.diagmicrobio.2018.07.007
0732-8893/

1. Introduction

Nowadays, *Candida* species play a pivot role among infectious diseases, being the fifth leading cause of hospital-acquired pathogen and the fourth leading cause of bloodstream infections (Sievert et al., 2013). More than 15 distinct *Candida* species infect humans; nevertheless, >90% of invasive candidiasis (IC) is caused by a few of them, such as *C. albicans, C. glabrata, C. tropicalis, C. parapsilosis*, and *C. krusei* (Pappas et al., 2015).

IC has become an increasing clinical problem, being a difficult-to-treat fungal infection within intensive care units and, more recently, also in medical wards (Prigitano et al., 2016). The difficulties in managing such cases often derive from delayed diagnosis due to lack of pathognomonic signs and to the poor sensitivity of the blood culture-based gold standard assay. Only about half of the patients affected by IC are blood culture positive, and also in these cases, the time to result is too long (2–7 days) with respect to the severity of the disease: indeed, a 12-h diagnostic delay is associated to a significant increase in lethality (Bassetti et al., 2007, 2013; Garey et al., 2006; Morrell et al., 2005). This leads to the administration of early empiric therapies using clinical scores based on risk stratification, such as candida colonization index (CCI) and candida score (CS). These clinical scores are highly sensitive, but they suffer from low specificity and low positive predictive value (PPV) (León et al., 2009; Pittet et al., 1994). As a consequence, empiric and preemptive therapies are often chosen; patients receive unnecessary, expensive, and toxic treatments whose negative effects also include promotion of fungal drug resistance (Ostrosky-Zeichner, 2012). To overcome these limitations, recent years have seen the introduction of noncultural microbiological assays to detect early markers of invasive fungal infections, such as fungal antigens/metabolites, fungal DNA, and even antifungal antibodies (León et al., 2012, 2016).

The (1 → 3)-β-D-glucan (BDG) is an important component of the cell wall of fungi; BDG detection in serum is one of the microbiological criteria for definition of IFI, according to the joint committee of the European Organization for Research and Treatment of Cancer-Mycosis Study Group (De Pauw et al., 2008).

Recently, some studies have shown the usefulness of another serological marker of IC, named *Candida albicans* germ tube antibodies (CAGTAs), whose performance may be enhanced when used in combination with other biomarkers of IC (León et al., 2012, 2016; Martínez-Jiménez et al., 2015; Martín-Mazuelos et al., 2015). Such a marker can be detected in serum by a commercially available kit (invasive candidiasis CAGTA immunofluorescence assay (IFA) immunoglobulin G [IgG]; Vircell Microbiologist S.L., Granada, Spain). By indirect immunofluorescence, CAGTA kit detects IgG antibodies against several superficial antigens of the germ tubes of *C. albicans*, including Hwp1 and others, as reported by Sáez-Rosón and Sevilla (2014) and by Laín et al. (2007). According to initial studies, serum CAGTA detection is suggestive of deep-seated candidiasis, where *Candida* hyphae have invaded different body tissues, eliciting a specific host antibody response (Brand, 2012; Laín et al., 2007).

Within this complex scenario, by a retrospective study, we evaluated the performance of CAGTA used both alone and in combination with BDG. By means of the CAGTA IFA kit, we tested 57 serum samples (29 from IC proven and 28 from control non-IC cases) that had been evaluated for BDG in a previous study (Pini et al., 2016); then, by a computer-assisted acquisition and elaboration of the fluorescence images, our results were quantified as arbitrary florescent units (AFU), and a cutoff value was established. Thus, we provide the first evidence that the protocol for CAGTA determination can be implemented by an objective reading that, in turn, allows an easy and rapid interpretation of the results. Moreover, we expand the evidence that CAGTA is a useful marker of IC, though special caution should be used because of its low sensitivity in non–*C. albicans* cases. As predicted, CAGTA performance increases in a relevant manner when used in combination with BDG.

2. Materials and methods

2.1. Definitions

As previously detailed, proven IC was defined as identification of *Candida* species in blood cultures or positivity in histopathology, cytopathology, or microscopic examination of normally sterile clinical samples obtained by biopsy or needle aspiration. Also, proven IC was defined upon recovery of *Candida* species by culture of a sample obtained (by means of a sterile procedure) from a normally sterile site showing a clinical or radiological abnormality consistent with an infectious disease (De Pauw et al., 2008).

2.2. Study population

The population of our retrospective, observational, monocentric study consisted of adult non-neutropenic patients hospitalized at the Azienda Ospedaliero-Universitaria (AOU) Policlinic of Modena in the period November 2011–January 2015; such a population had been enrolled in a prospective observational study recently published by our group (Pini et al., 2016).

All the sera from those patients were stored and anonymized. In particular, we worked on 57 samples previously tested for BDG, 29 sera from IC episodes (IC group) and 28 from non-IC control episodes (non-IC group). In particular, 27/29 IC sera had positive blood culture for *Candida* spp., and 2/29 had intrasurgery intestinal biopsy positive for fungal hyphae; 12/29 of the IC samples belonged to patients with deep-seated candidiasis (7 from tertiary peritonitis and 5 from chronic disseminated candidiasis) and 15/29 from non–deep-seated candidiasis. The CTRL group (28 sera) included 12 sera from non-IC high-risk patients colonized with *Candida* spp. and 16 sera from non-IC and noncolonized patients.

2.3. Samples

Serum samples had been obtained from blood after centrifugation at 5000 rpm for 5 min. The samples were stored at −80 °C until CAGTA analysis. All the sera included in the present study had been previously evaluated for BDG levels, as detailed elsewhere (Pini et al., 2016), by the commercial Fungitell® assay (Associates of Cape Cod Inc., Falmouth, MA).

2.4. CAGTA assay

The *Candida albicans* germ-tube antibody (CAGTA; invasive candidiasis immunofluorescence assay [IgG]; Vircell Microbiologist S.L., Granada, Spain) is a commercially available indirect immunofluorescence test that detects antibodies against superficial antigens of the germ-tubes of *C. albicans* in human serum/plasma. Serum samples were processed according to the manufacturer's instructions. At first, samples were clarified by centrifugation (700 RCF, 2′) and diluted 1:4 in phosphate-buffered saline (PBS); subsequently, 20 μL of the resulting solution was added to 80 μL of Vircell Candida sorbent (*C. albicans* yeast phase heat-inactivated cells used to remove antiyeast antibodies from the serum). The solution obtained (1:20 dilution) was incubated and centrifuged (700 RCF, 5′); then, 50 μL of supernatant were taken, and 4 dilutions were prepared using PBS: 1:40, 1:80 1:160, and 1:320. After that, an aliquot (20 μL) of each dilution was deposited into the slide wells, each one containing *Candida albicans* mycelial-phase cells, fixed with acetone. Then, each well was gently washed, and 20 μL of anti-human IgG antibodies, conjugated with fluorescein, were added. After additional washes, the slides were observed at the epifluorescence microscope using a 400× lens. It should be noted that the acquisition parameters (exposure times, etc.) remained unchanged for all the experiments. From here, the reading of each sample was accomplished in 2 ways. First, a standard reading was performed: according to the

manufacturer, a qualitative interpretation of the results was given by examination of the 1:160 serum dilutions; a serum sample was given as positive when an "apple-green fluorescence" appeared on the mycelial phase of *Candida* and, at the same time, a red fluorescence was evident on the yeast forms. Second, an alternative reading was performed: the fluorescence images in each sample were acquired (at least 5 photos/sample) by a dedicated software; then, the green fluorescence around the mycelial forms was selected by a dedicated function, captured, and expressed as arbitrary fluorescence units (AFU); aspecific fluorescence was excluded by visual control, and in any case, blastospores never resulted fluorescent. The data obtained were refined by calculating the mean fluorescence of at least 100 hyphae/sample. This procedure, as detailed below in Section 2.5, allowed us to build an ROC curve, thus establishing an intensity of fluorescence cut-off value.

2.5. Statistical analysis

Sensitivity, specificity, PPVs, negative predictive values (NPVs), and accuracy were calculated, and the 95% confidence intervals (95% CI) for binomial variables were estimated using Clopper–Pearson exact distribution or normal distribution according to the number of observations.

To assess differences, the χ^2 or Fisher's exact test was used for independent binomial variables according to the number of observations; the null hypothesis was rejected for a P value < 0.05.

Diagnostic performance was evaluated by ROC curve analysis, estimating the areas under the curve and their 95% CI; the best cutoff was established using Youden index.

3. Results

3.1. Characteristics of the serum samples to be assessed

Two groups of serum samples, previously stocked and analyzed as recently described (Pini et al., 2016), named "IC" and "non-IC," were included in the present study. Their clinical and microbiological characteristics are depicted in Table 1. In particular, the IC group consisted of 29 serum samples, the ICU ward being the most represented ($n = 21$ sera); other wards included pneumology ($n = 4$), gastroenterology ($n = 2$), surgery ($n = 1$), and infectious diseases ($n = 1$). As detailed also in Table 1, *C. albicans* was the most frequently isolated species (48%), followed by *C. glabrata* (24%); other isolates (10%) included *C. tropicalis*, *C. parapsilosis*, and *C. pelliculosa.* In the IC cases documented by biopsy, the identification at the species level was not possible.

Table 1
Clinical and microbiologic characteristics of the enrolled patients.

	All samples $n = 57$ (%)	IC samples $n = 29$ (%)	Non-IC samples $n = 28$ (%)
Clinical characteristics			
Ward			
Hematology	5 (8.8)	0 (0.0)	5 (17.9)
Intensive care unit	44 (77.2)	21 (72.4)	23 (82.1)
Pneumology subintensive unit	4 (7.0)	4 (13.8)	0 (0.0)
Infectivology	1 (1.8)	1 (3.4)	0 (0.0)
Gastroenterology	2 (3.5)	2 (6.9)	0 (0.0)
Surgery	1 (1.8)	1 (3.4)	0 (0.0)
Underlying disease			
Surgical	18 (31.6)	7 (24.1)	11 (39.3)
Medical	39 (68.4)	22 (75.9)	17 (60.7)
Microbiological characteristics			
Yeasts (blood culture)			
C. albicans	14 (24.6)	14 (48.3)	0 (0.0)
C. glabrata	7 (12.3)	7 (24.1)	0 (0.0)
C. parapsilosis	1 (1.8)	1 (3.4)	0 (0.0)
C. tropicalis	1 (1.8)	1 (3.4)	0 (0.0)
C. pelliculosa	1 (1.8)	1 (3.4)	0 (0.0)
C. glabrata + *R. mucillaginosa*	1 (1.8)	1 (3.4)	0 (0.0)
Bacteria (blood culture)			
A. baumanii	3 (5.3)	3 (10.3)	0 (0.0)
E. faecalis	1 (1.8)	1 (3.4)	0 (0.0)
E. faecium	5 (8.8)	0 (0.0)	5 (17.9)
S. pneumoniae	2 (3.5)	0 (0.0)	2 (7.1)
P. aeruginosa + *A. baumanii*	2 (3.5)	0 (0.0)	2 (7.1)
Biopsy positive for fungal hyphae	2 (3.5)	2 (6.9)	0 (0.0)

In the non-IC group, including 28 serum samples, 24 sera were derived from ICU patients; the remaining 4 sera were obtained from individuals of the hematology ward. Furthermore, among the non-IC serum samples, 9 sera were derived from septic patients (*E. faecium* $n = 5$, *S. pneumoniae* $n = 2$, *P. aeruginosa* + *A. baumannii* $n = 2$), while the other 19 samples had returned negative blood cultures.

3.2. Standard vs alternative CAGTA reading and establishment of an AFU cutoff value

All the sera were processed for CAGTA using *Candida* precoated slides and reagents as detailed in the kit procedure. Following the immunofluorescence labeling, the slides were evaluated in parallel: by a standard operator-dependent optical reading and by an alternative computer-assisted capture/elaboration of the fluorescence images. This second approach was aimed at the objective quantification of the signal intensity in each sample. As detailed in Materials and Methods (-Section 2), the results were expressed as mean AFU ± SD of at least 100 hyphae/sample. An ROC curve analysis was then drawn (Fig. 1), and an interpretative cutoff was established (25.8 AFU). It should be noted that the 2 reading procedures provided comparable information in terms of positive/negative CAGTA samples. Interestingly, the AFU reading enabled a semiquantitative evaluation of the samples and an objective interpretation of the results based on the cutoff value.

3.3. Performances of CAGTA and BDG

According to the classification in IC and non-IC groups, the performance of CAGTA and BDG, used alone and in combination, was assessed (Table 2). BDG demonstrated a sensitivity higher than CAGTA (89.7% versus 51.7%), and the association of the 2 markers increased such a parameter up to 96.6%. When comparing the specificity, CAGTA performed better than BDG (89.3% versus 75.0%), and the association of the 2 markers maintained the specificity at 71.4%. The best overall accuracy (84.2%) was obtained by the association of the 2 markers followed by that of BDG (82.5%) and CAGTA (70.2%) used alone. As indirectly indicated by the numbers in square parentheses, the false-negative results

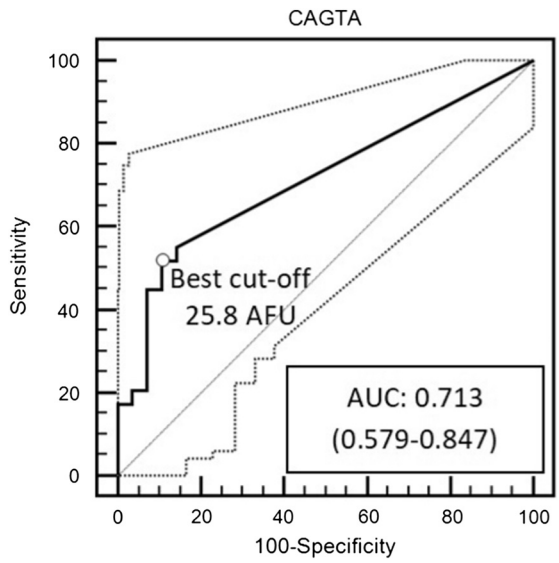

Fig. 1. CAGTA fluorescence estimation ROC curves (dotted lines 95% IC).

Table 2
Overall performance of CAGTA and BDG.

Marker	Test result % (95% CI) [no. of samples with result[a]/total no. of tested samples]				
	Sensitivity	Specificity	PPV	NPV	Accuracy
CAGTA $n = 57$	51.7 (33.5–69.9) [15/29]	89.3 (71.8–97.7) [25/28]	83.3 (58.6–96.4) [15/18]	65.8 (50.7–80.9) [25/38]	70.2 (58.3–82.1) [40/57]
BDG $n = 57$	89.7 (72.6–97.8) [26/29]	75.0 (59.0–91.0) [21/28]	78.8 (64.8–92.7) [26/33]	87.5 (67.6–97.3) [21/24]	82.5 (72.6–92.3) [47/57]
CAGTA or BDG positive $n = 57$	96.6 (82.2–99.9) [28/29]	71.4 (54.7–88.2) [20/28]	77.8 (64.2–91.4) [28/36]	95.2 (76.2–99.9) [20/21]	84.2 (74.7–93.7) [48/57]

[a] Number of samples with true result with respect to each tested parameter.

with CAGTA were higher (13) than with BDG alone (3) or CAGTA plus BDG (1); on the contrary, the false-positive results were only 3 in CAGTA, 7 in BDG, and 8 in the association of the 2.

3.4. CAGTA and BG performance by a subgroup analysis

The results were further analyzed; in particular, the performances of CAGTA, BDG, and their combination were evaluated upon clustering sera in subgroups, as detailed in Table 3. First, taking into account the *Candida* species, we compared the sensitivity of CAGTA and BDG in *C. albicans* IC group versus non *C. albicans* IC group; the highest difference was observed for CAGTA that demonstrated a sensitivity of 85.7% for *C. albicans* IC and only of 9.1% for non–*C. albicans* IC (P value = 0.000213). Second, we compared the sensitivity of CAGTA and BDG considering the type of candidiasis. We found that CAGTA showed the highest variation since the sensitivity ranged from 66.7% in deep-seated to 41.2% in non–deep-seated candidiasis group. In addition, 100% sensitivity was obtained for deep-seated candidiasis due to *C. albicans* (data not shown). Moreover, the association of the 2 markers was able to increase the sensitivity to 100% in deep-seated candidiasis and up to 94.1% in non–deep-seated group. Finally, when evaluating the influence of colonization, BDG demonstrated the most drastic decrease in specificity that dropped from 87.5% in noncolonized patients to 58.3% in colonized patients.

4. Discussion

In clinical settings, the detection of early markers for prompt identification of IC cases becomes very important as a consequence of the blood culture (gold standard for diagnosis) low sensitivity (as established by autopsy cases studies (Kami et al., 2002; Obayashi et al., 2008)) and because of the long time to result, which normally ranges from 2 to 7 days. It is well known that even a mere 12 hours of diagnostic delay can be associated to a significant increase in lethality (Bassetti et al., 2007, 2013; Garey et al., 2006; Morrell et al., 2005).

In this study, we have evaluated the usefulness of CAGTA maker and its association with BDG. The commercially available test for CAGTA detection is an indirect immunofluorescence assay developed for the diagnosis of IC. CAGTAs are produced specifically during tissue invasion by fungal hyphae, when a deep-seated involvement occurs. From here, the rationale for searching such antibodies. Notwithstanding the several studies published on this topic, the diffusion and use of the CAGTA test in clinical microbiological laboratories remain limited. The assay needs an operator-dependent reading and is characterized by quite low sensitivity values, ranging from 53% to 84% (León et al., 2016; Martínez-Jiménez et al., 2015; Martín-Mazuelos et al., 2015; Moragues et al., 2004). Our present results confirm such overall low sensitivity (52%). Interestingly, by the subgroup analysis, we demonstrate a major difference in the sensitivity of CAGTA when detecting *C. albicans* candidiasis with respect to non–*C. albicans* cases (86% versus 9%, respectively). Differently, it should be noted that, as expected, the performance of BDG is not affected by the exclusion of these samples, in line with the fact that BDG is a pan-fungal marker. Previous studies had similarly reported a decrease in CAGTA sensitivity in non-*C. albicans* infections (Martínez-Jiménez et al., 2015); yet, the rate of positivity varies to some extent; in particular, all our 7 episodes due to *C. glabrata* were negative, while a previous study analyzing 4 *C. glabrata* episodes reports a sensitivity of 75% (Martínez-Jiménez et al., 2015). Such *Candida* species-related variation in sensitivity of CAGTA may be explained by the fact that *C. glabrata,* for instance, is unable to produce either hyphae or pseudohyphae (Kaur et al., 2005; Rodrigues et al., 2017); thus, it is reasonable that no or low anti-germ tube specific antibodies, such as CAGTAs, are detectable in sera from patients with *C. glabrata* infections. Nevertheless, we cannot exclude that CAGTA assay may also detect antibodies cross-reacting with *Candida* nonalbicans antigens,via shared homology sites, thus allowing positive results even in IC by *Candida* spp. not producing hyphal forms.

In any case, further investigations are needed to clarify this issue since limited data are available so far defining CAGTA performance on different *Candida* species (Kaur et al., 2005; Rodrigues et al., 2017 and present study).

When clustering Candida infections on the bases of clinical features, we demonstrate the best performance of CAGTA in deep-seated cases, with the sensitivity reaching 100% in episodes due to *C. albicans*. Also, this observation is in accordance with that previously observed by other authors (Martínez-Jiménez et al., 2014).

Table 3
Performance of BDG and CAGTA by a subgroup analysis.

Parameters	Test result % (95% CI) [no. of samples with result[a]/total no. of tested samples]		
	BDG	CAGTA	BDG or CAGTA positive
Sensitivity in samples from:			
C. albicans IC	92.9 (66.1–99.8) [13/14]	85.7 (57.2–98.2) [12/14]*	100.0 (76.8–100.0) [14/14]
Non-*albicans* IC	90.9 (58.7–99.8) [10/11]	9.1 (0.2–41.3) [1/11]*	90.9 (58.7–99.8) [10/11]
Sensitivity in samples from:			
Deep-seated candidiasis	91.7 (61.5–99.8) [11/12]	66.7 (34.9–90.1) [8/12]	100.0 (73.5–100.0) [12/12]
Non–deep-seated candidiasis	88.2 (63.6–98.5) [15/17]	41.2 (17.8–64.6) [7/17]	94.1 (71.3–99.9) [16/17]
Specificity in samples from:			
Colonized patients	58.3 (27.7–84.8) [7/12]	83.3 (51.6–97.9) [10/12]	58.3 (27.7–84.8) [7/12]
Non-colonized patients	87.5 (61.7–98.4) [14/16]	93.8 (69.8–99.8) [15/16]	81.3 (54.4–96.0) [13/16]

[a] Number of samples with true result with respect to each tested parameter.
* P value = 0.000213.

By the present work, we show that CAGTA performs better than BDG in terms of specificity; in particular and as predicted, BDG is more influenced than CAGTA by *Candida* colonization of patients, with an observed drop in specificity from 86% in noncolonized group to 58% in the colonized group.

Finally, it is worth noting that the association of the 2 markers results in an overall sensitivity of 97% and in the highest overall accuracy, namely, 84%. Undoubtedly, the concomitant use of CAGTA and BDG should be recommended in high-risk patients where conventional diagnostic tests are either time consuming or have low sensitivity; furthermore, the local epidemiology would drive the use of CAGTA when the *C. albicans* infections are prevalent.

5. Conclusion

CAGTA is a useful IC marker whose sensitivity and overall accuracy greatly increase when used in association with BDG; moreover, the high sensitivity of CAGTA in detecting *C. albicans* deep-seated infections underlines the utility of such marker in clinical settings such ICU and surgical patients to promptly recognize deep-seated involvement in cases where conventional diagnostic tools often fail. Importantly, the best performance of CAGTA occurs in *C. albicans* infections, which in most clinical settings still remain the prevalently isolated *Candida* species. Furthermore, we propose an original procedure to read the CAGTA results; the computer-assisted fluorescence assessment, demonstrating a great agreement with the operator-dependent standard reading, opens to a semiquantitative analysis of the CAGTA marker, thus facilitating standardization of the procedure and interpretation the results.

Conflict of interest

The authors declare that they have no conflict of interest.

Ethical approval

All procedures performed in the study involving human participants were in accordance with the ethical standards of the institutional research committee (n. 59/17) and with the 1964 Helsinki declaration and its later amendments or comparable ethical standards.

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