mycoses
Diagnosis, Therapy and Prophylaxis of Fungal Diseases

Original article

Enzymatic activity profile of a Brazilian culture collection of *Candida albicans* isolated from diabetics and non-diabetics with oral candidiasis

Paula Volpato Sanitá, Chaiene Evelin Zago, Ana Cláudia Pavarina, Janaina Habib Jorge, Ana Lúcia Machado and Carlos Eduardo Vergani

Department of Dental Materials and Prosthodontics, Araraquara Dental School, UNESP – Univ Estadual Paulista. Rua Humaitá, Araraquara, Sao Paulo, Brazil

Summary

The secretion of hydrolytic enzymes is a fundamental virulence factor of *Candida albicans* to develop disease. The objective of this study was to characterise the virulence of 148 clinical isolates of *C. albicans* from oral candidiasis by assessing the expression of phospholipase (PL) and secreted aspartyl proteinase (SAP). Isolates were obtained from healthy subjects (HS) and diabetics (DOC) and non-diabetics with oral candidiasis (NDOC). An aliquot (5 μl) of each cell suspension was inoculated on PL and SAP agar plates and incubated. Enzymes secretion was detected by the formation of an opaque halo around the colonies and enzymatic activity (PZ) was determined by the ratio between colony diameter and colony diameter plus the halo zone. Statistical comparisons were made by a one-way ANOVA followed by Tukey's *post hoc* test ($\alpha = 0.05$). The clinical sources of *C. albicans* had significant effect ($P < 0.001$) on the PZ values of both enzymes. For PL, clinical isolates from NDOC and DOC had highest enzymatic activity than those from HS ($P < 0.05$), with no significant differences between them ($P = 0.506$). For SAP, *C. albicans* from NDOC showed the lower enzymatic activity ($P < 0.001$). There were no significant differences between isolates from HS and DOC ($P = 0.7051$). *C. albicans* isolates from NDOC and DOC patients showed an increased production of PL.

Key words: *Candida albicans*, phospholipase, secreted aspartyl proteinase, oral candidiasis, diabetes mellitus.

Introduction

Among all candidal infections, oral candidiasis is the most common form and especially affects elderly individuals.[1–3] There are several local and host factors that are known to predispose oral candidiasis. *Candida* spp. have the ability to adhere to the denture tissue surface, which acts as a reservoir of pathogens, favouring yeast proliferation and enhancing their infective potential.[1–3] Another host factor that may promote the oral carriage of *Candida* is the presence of diabetes mellitus.[4] In diabetic patients, high salivary glucose levels favour yeast growth due to increased number of available receptors for *Candida*.[5,6] Consequently, buccal cells from diabetic patients have shown an increased adherence of *C. albicans* compared to buccal cells from non-diabetics.[7,8] The oral candidiasis progress is also faster and more severe in diabetics, as they possess a deficient immune system that predispose to *Candida* colonisation.[4,6] Consequently, a significantly higher incidence of *Candida* infection[9] and increased levels of *Candida* spp. have been found in diabetic patients,[9–11] especially those wearing removable denture.[2,12]

Correspondence: C. E. Vergani, Rua Humaitá, 1680, Araraquara, SP, Brazil, CEP 14801903.
Tel.: +55 16 33016542. Fax: +55 16 33016406.
E-mail: vergani@foar.unesp.br

Submitted for publication 30 July 2013
Revised 20 October 2013
Accepted for publication 19 November 2013

doi:10.1111/myc.12162

Besides the local and host factors that predispose oral candidiasis, the infective ability of the *C. albicans* yeasts also depends on several virulence factors. This species is the most virulent and pervasive of all the *Candida* spp.,[13] which is the reason for its preeminent position in the hierarchy of prevalence.[1–3,12] Among the virulence factors of *C. albicans*, adhesion to host surfaces (epithelial cells and denture surfaces), as well as biofilm formation, are considered the first step to initiate *Candida* infection.[14] The ability of *C. albicans* to produce extracellular hydrolytic enzymes, such as phospholipases (PLs) and secreted aspartyl proteinases (SAPs), is another important virulence factor.[13] PLs, which are concentrated mostly in the tips of the hyphae,[15] have an active role in the invasion of lesions in the host tissue, as this enzyme causes the rupture of the epithelial cell membrane and permit the penetration of the fungi cell into the cytoplasm.[16] SAPs induce the degradation of a large variety of host proteins, increasing the ability of the fungi to colonise and penetrate tissues.[17] Therefore, these enzymes together play an important role in the tissue invasion process and, consequently, in the pathogenicity of *C. albicans*.

The literature contains substantial data showing that the expression of these different extracellular hydrolytic enzymes has a large variation intra and inter species of *Candida*.[11,18–28] Moreover, several studies focused on the production of PLs and SAPs by clinical isolates of *Candida* species obtained from different patients, such as those healthy with normal oral mucosa or oral candidiasis,[19,20,23,24] or those with systemic diseases, such as diabetes mellitus.[21,22,26] Nevertheless, to the authors' knowledge, there has been only one investigation[11] that compared the secretion of these enzymes by *Candida* yeasts isolated from diabetic and non-diabetic denture wearers with oral candidiasis. The characterisation of the virulence factors of the strains responsible for a particular infection, such as the enzymatic activity profile, is essential to determine strategies for prevention, control and treatment of this type of fungal infection. Thus, the central aim of this study was to characterise the virulence of a Brazilian culture collection of 148 clinical isolates of *C. albicans* from oral candidiasis by assessing the expression of two extracellular hydrolytic enzymes, PL and SAP. In addition, this study aimed at assessing the different rates in the secretion of candidal histolytic enzymes among clinical isolates of *C. albicans* from healthy subjects and from diabetic and non-diabetic patients with oral candidiasis.

Materials and Methods

Clinical isolates of C. albicans

This study evaluated 148 clinical isolates of *C. albicans* from oral candidiasis obtained from the yeast culture collection of the Laboratório de Microbiologia Aplicada – UNESP – Univ Estadual Paulista, Araraquara, SP, Brazil. These isolates were previously obtained from the tissue surfaces of the dentures of edentulous patients with different systemic/oral conditions, in a previous investigation.[12] Fifty-eight clinical isolates were obtained from healthy subjects (HS), who were non-diabetics without oral candidiasis; 58 clinical isolates were obtained from non-diabetics with oral candidiasis (NDOC); and 32 clinical isolates were obtained from diabetic patients with oral candidiasis (DOC).[12] All subjects with oral candidiasis were diagnosed with denture stomatitis according to criteria proposed by Newton [29]. Denture sample was made using a sterile oral swab.[12] All demographic characteristics of the patients and the distribution of risk factors for denture stomatitis among them (age of dentures, smoking habit) are available in our previous investigation.[12] All procedures followed the criteria of Resolution 196/96 of the Brazilian Health Ministry and the study was approved by the Ethics Committee of the Araraquara Dental School, UNESP – Univ Estadual Paulista.

Several *Candida* species were collected and identified during sample phase, including *C. albicans*, *C. glabrata*, *C. tropicalis* and *C. krusei*.[12] The yeast identification procedures for *Candida* spp. included the presumptive identification on CHROMAgar Candida, the micromorphological characteristics on corn meal agar with polysorbate 80 (Tween 80; Sigma-Aldrich Co LLC, St Louis, MO, USA) for the production of hyphae and chlamidoconidia and the assimilation of a variety of carbon and nitrogen sources using the ID32C yeast identification system (bioMérieux, Marcy-l'Etoile, France).[12] The hypertonic Sabouraud broth test[30] was performed to discriminate *C albicans* and *C dubliniensis*, when necessary. Two reference strains were used as positive controls: *C. albicans* ATCC 90028 and *C. albicans* wild-type strain SC 5314. All isolates were maintained in Yeast-Peptone-Glucose (1% yeast extract, 2% Bacto peptone, 2% D-glucose and 2% agar) medium and frozen at −70 °C until further use.

Culture conditions and standardisation

The microorganisms were subcultured onto Sabouraud Dextrose Agar (SDA - Acumedia Manufactures Inc.,

Baltimore, MD, USA) plates supplemented with chloramphenicol (0.05 g l^{-1}) and incubated at 37 °C for 24–48 h. To prepare the yeast inoculums, 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 mmol l^{-1} glucose and incubated at 37 °C overnight in an orbital shaker (75 rpm). Cells of the resultant cultures were harvested and washed twice with phosphate-buffered saline (PBS, pH 7.2) at 5000 × g for 5 min. Washed microorganisms were resuspended in sterile distilled water and spectrophotometrically standardised at an optical density at 520 nm (Biospectro, Equipar Ltda, Curitiba, PR, Brazil) to a final concentration of 10^8 cfu ml^{-1}.[25]

Phospholipase secretion

Phospholipase production testing was performed according to the methodology of Price *et al.* [31] 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). Potassium tellurite was used in the egg yolk emulsion to improve the visualisation of the halos of precipitation and the accuracy of measurements. Then, three aliquots of 5 µl of each cell suspension of 10^8 cfu ml^{-1} were inoculated on the egg yolk medium and plates were incubated at 37 °C for 7 days.[25] After incubation, the diameter of hyaline zones around the colonies and the diameter of the colonies were measured using a digital caliper.[24] The phospholipase activity (PZ-PL value) was measured in terms of the ratio of the diameter of the colony to the total diameter of colony plus zone of precipitation. PZ-PL was also categorised as follows: PZ-PL < 0.64 (very high), 0.64 ≤ PZ-PL < 1 (high) and PZ-PL = 1 (negative).[11] Each isolate was tested in triplicate in three independent experiments and the PL activity value was recorded as the average of the three measurements.

Secreted aspartyl proteinase secretion

Secreted aspartyl proteinase production testing was performed according to the methodology of Rüchel [32] with a few modifications. The test medium consisted of agar plates containing bovine serum albumin. The agar medium was prepared using 20 g of dextrose, 1 g of potassium phosphate, 0.5 g of magnesium sulphate and 15 g of agar in 1 l of distilled water. This solution was autoclaved and cooled down to 50 °C. The bovine serum albumin medium was prepared using 2 g of albumin, 0.2 g of riboflavin, 0.4 g of nicotinic acid and 0.4 g of thiamine hydrochloride in 1 l of distilled water. This solution was sterilised by filtration in a 0.22-µm membrane and mixed with the agar medium. Then, three aliquots of 5 µl of each cell suspension of 10^8 cfu ml^{-1} were inoculated on the plates, which were incubated at 37 °C for 7 days.[25] The proteolysis activity in the plates was visualised 24–48 h after staining with amido black solution (amido black 0.5%, glacial acetic acid 49.5%, distilled water 50%).[20] The diameters of colonies and colonies plus zone of precipitation were measured using a digital caliper and the proteinase activity (PZ-SAP) was determined according to the method used in the PL testing. PZ-SAP was also categorised as follows: PZ-SAP<0.64 (very high), 0.64 ≤ PZ-SAP < 1 (high) and PZ-SAP = 1 (negative).[11] Each isolate was tested in triplicate in three independent experiments and the PL activity value was recorded as the average of the three measurements. It is important to mention that all measurements were performed by the same calibrated operator.

Statistical analysis

There were two variable of interest in this study, which were the PL and SAP activities (PZ-PL and PZ-SAP values, respectively). The factor analysed was the clinical source of the isolates of *C. albicans*: HS, NDOC and DOC. PL and SAP activities were compared according to these clinical sources of the isolates of *C. albicans*. All PL/SAP negative strains were excluded from these analyses. As data satisfied the assumptions of normality and homogeneity of variance, parametric analyses were conducted with a confidential level of 95%. One-way ANOVAs followed by Tukey's *post hoc* test were used to compare the PZ-PL and PZ-SAP values among the three sources of the clinical isolates of *C. albicans*.

Results

In general, from the 148 clinical isolates evaluated in this study, 106 (71.6%) were PL positive and 138 (93.2%) were SAP positive. As expected, the ATCC reference strains were also PL and SAP positive, providing further confidence in the reliability of the assays.

The enzymatic activity values of *C. albicans* ATCC were 0.78 for PZ-PL and 0.58 for PZ-SAP. The enzymatic activity values of *C. albicans* wild-type strain were 0.70 for PZ-PL and 0.73 for PZ-SAP.

The categorised results showed that most *C. albicans* isolates produced high PL activity and 42 clinical isolates did not present any activity. Considering SAP results, categorised data showed that most *C. albicans* isolates produced very high SAP activity (118 isolates), 20 isolates exhibited high SAP activity and other 10 did not present any activity. The descriptive statistics of the enzymatic activity profiles of all clinical isolates evaluated in this study is shown in Table 1. Illustrative images are shown in Fig 1.

One-way ANOVA showed that the different clinical sources of *C. albicans* had significant effect ($P < 0.0001$) on the PZ values of both enzymes. The PZ-PL values of the positive strains ranged from 0.71 to 0.99, 0.67 to 0.99 and 0.70 to 0.99 for HS, NDOC and DOC clinical isolates respectively. The clinical isolates from NDOC and DOC had higher enzymatic activity of PL, expressed by lower PZ-PL values, than those from HS ($P = 0.0250$ and $P < 0.001$ respectively), with no significant differences ($P = 0.506$) between them (Table 2). For SAP activity, the PZ-SAP values of the positive strains ranged from 0.18 to 0.98, 0.36 to 0.96 and 0.22 to 0.87 for HS, NDOC and DOC clinical isolates respectively. By contrast, *C. albicans* from NDOC showed the lower SAP activity ($P < 0.001$). There were no significant differences ($P = 0.7051$) between clinical isolates from HS and DOC (Table 2).

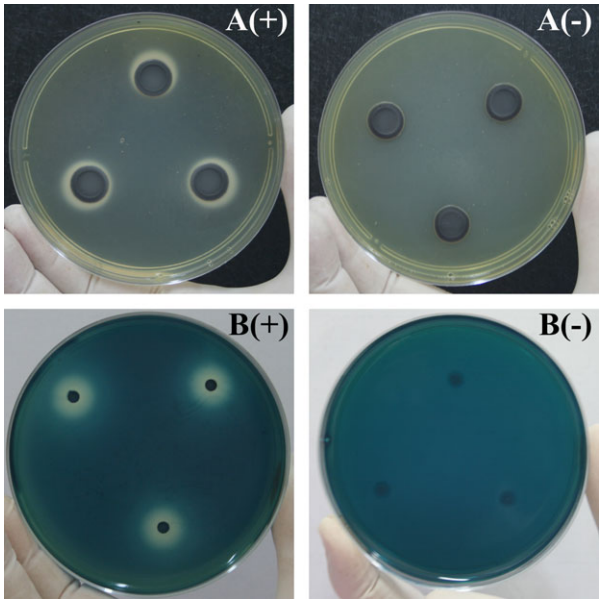

Fig 1 Representative images of PL positive/negative (A) and SAP positive/negative (B) activities of a clinical isolate of *C. albicans.*

Table 2 Mean values of PZ-PL and PZ-SAP and standard deviation considering the three clinical sources of *C. albicans.*

Source of clinical isolate	PZ-PL	PZ-SAP
HS	0.9236 (0.065)^{A}	0.4084 (0.173)^{A}
NDOC	0.8385 (0.088)^{B}	0.5312 (0.140)^{B}
DOC	0.8632 (0.093)^{B}	0.3800 (0.159)^{A}

HS, healthy subjects; NDOC, non-diabetics oral candidiasis; DOC. diabetics oral candidiasis; PZ-PL, phospholipase activity; PZ-SAP, SAP activity.

^{A,B}In columns, values with the same uppercase letters were not statistically different ($P > 0.05$).

Discussion

This study investigated the enzymatic activity profile of a Brazilian culture collection of 148 clinical isolates of *C. albicans* isolated from healthy subjects and diabetics and NDOC and the influence of these oral/systemic conditions in the virulence of the microorganisms In accordance with the results, PL and SAP activities were observed in an elevated percentage of the clinical isolates of *C. albicans* (71.6% and 93.2% respectively). These findings are partially in agreement with those from previous investigations. D'Eça Júnior *et al.* [27] also found a similar percentage of PL positive activity (76.6%) among clinical isolates of *C. albicans*, but a

Table 1 Number and percentage of *Candida albicans* isolates from oral candidiasis with negative, high and very high hydrolytic enzymatic activity profiles according to their clinical source.

	PL			SAP		
Activity	HS ($n = 58$)	NDOC ($n = 58$)	DOC ($n = 32$)	HS ($n = 58$)	NDOC ($n = 58$)	DOC ($n = 32$)
Negative	19 (32.7%)	10 (17.2%)	13 (40.6%)	7 (12.1%)	2 (3.4%)	1 (3.1%)
High	39 (67.3%)	48 (82.8)	19 (59.4%)	5 (8.6%)	12 (20.7%)	3 (9.4%)
Very high	–	–	–	46 (79.3%)	44 (75.9%)	28 (87.5%)

HS, healthy subjects; NDOC, non-diabetics oral candidiasis; DOC, diabetics oral candidiasis.

lower proportion of SAP-positive isolates (48.9%) was observed by them. Others found percentages greater than 90% for both PL and SAP activity among clinical isolates of *C. albicans*.[18,20,23] The differences in findings among studies could be related to the large variation intra- and interspecies of *Candida*, as shown in several reports.[11,18–27] In addition, some differences in the methodology may have played a role in these discrepancies. The incubation period is an important factor, as distinct halos of precipitation may not be developed in reduced incubation periods.[15] Thus, an incubation period of 7 days was used in this study for both PL and SAP activity tests. Qualitative variations in the culture media used, such as protein source, as well as the quantity of other ingredients and the pH of the solutions[15] may also have influenced the differences in results among studies. An important study of Samaranayake *et al.* [15] showed that PL activity of *C. albicans* isolates was suppressed by increasing concentrations of sucrose and galactose in the media. In addition, the authors observed that PL secretion was limited to a narrow pH range of about pH 3.6–4.7.[15] The technique used to measure the halo of precipitation of the enzymatic activity is also of great importance and may be the most relevant factor. While some authors reported that they used a digital caliper[24] or a computerised image analysis system[22] to determine the diameter of the colonies and precipitation zone, this information was omitted from most of the studies.[11,19,20,23,25,26] To improve the visualisation of the halos of precipitation and the accuracy of measurements, an egg yolk emulsion with potassium tellurite was used in the PL culture medium, so that yeast black colonies with a white halo were analysed (Fig 1). For SAP analyses, the agar plates were stained with amido black solution (Fig 1) leaving the halo unstained.[20]

The results of this study showed an increased PL activity among the clinical strains of *C. albicans* isolated from oral candidiasis patients (Table 2). This finding is in accordance with a previous study that found that PL production by *C. albicans* was significantly associated with the presence of denture stomatitis.[33] This demonstrates that PL production plays an important role in the development of this oral infection. It is known that *C. albicans* hyphal formation is a critical determinant in invasive pathogenesis. Once in the hyphal form, host epithelial layers can be pierced, resulting in tissue penetration, a crucial step in the initiation of infection.[34] PLs are concentrated mostly in the tips of the hyphae[15] and attack phospholipids common in all cell membranes.[16] It is plausible to assume that the damage to the integrity of the epithelial cells caused by the action of this enzyme may favour the access of the growing tips of the *C. albicans* hyphae to the deeper layers of epithelium. Thus, considering the infective role of PL in the infection process, it could be considered a target for antifungal therapy. In this context, the use of inhibitors of the PL of *C. albicans* might turn out to be particularly rewarding for oral candidiasis treatment and prevention.[35,36]

It is important to mention that an increased PL activity among the *C. albicans* isolates from oral candidiasis patients was observed, independent of whether they presented diabetes mellitus or not (Table 2). This result is in accordance with previous data showing that there were no significant differences in PL activity neither between *C. albicans* isolates from diabetic and non-diabetic patients [11,22] nor between *C. albicans* isolates from diabetic patients with different levels of glucose control.[26] In this study, all *C. albicans* clinical isolates from diabetic patients were obtained from well-controlled type 2 diabetic patients.[12] Considering the results above, it is possible that individuals harbouring these pathogenic and more virulent microorganisms are more susceptible to develop clinical manifestations of oral candidiasis when an opportunity arises, regardless the presence of systemic diseases.

Besides the presence of PL, it has been suggested that SAPs activity plays an active role in the invasion of lesions in the host tissue.[37] SAPs exhibit broad substrate specificity and are able to degrade many human proteins found at lesion sites, such as albumin, haemoglobin, keratin, collagen, mucin and secretory immunoglobulin A.[17,38,39] Degradation of these proteins *in vivo* may help *C. albicans* to acquire essential nitrogen for growth and to attach to and penetrate the oral mucosa.[37] In addition, the degradation of secretory immunoglobulin A is another strategy used by *C. albicans* to evade the immune response.[37] Thus, the presence of this enzyme may increase the ability of the fungi to colonise and penetrate tissues.[17] Our results showed that whereas oral *C. albicans* isolates from non-diabetics volunteers suffering from oral candidiasis (NDOC) showed relatively low SAP activity, clinical isolates from DOC produced relatively high amounts of this enzyme (Table 2). Other studies corroborate these results and showed that *C. albicans* isolated from diabetics with[11] or without[22,26] oral candidiasis had higher SAP activity then those strains from non-diabetic individuals.

Another surprising finding of the present investigation was the higher SAP activity demonstrated by the *C. albicans* isolated from control patients when

compared to that of the isolates from NDOC (Table 2). Contrasting results have been found in the literature. Data from previous reports showed that *C. albicans* obtained from oral candidiasis patients presented higher SAP activity compared to strains from healthy oral mucosa individuals.[19,20] By contrast, others have shown that the SAP activity did not differ considerably between patients with or without denture stomatitis.[23,33] In fact, Marcos-Arias *et al.*[33] did not find correlation between SAP production by *Candida* spp. and the presence of denture stomatitis. We expected to find differences in the SAP activity favouring the clinical isolates from oral candidiasis patients, as we found for PL activity, and this difference could be related to intra- and interstrain heterogeneity[11,18–27] or to methodological differences. Furthermore, another possible explanation of this result is the fact that both, PL and SAP, are active only at low pH.[15,40] In denture wearer patients, the pH underneath dentures may decline to levels lower than pH 4.0,[41] thus favouring enzyme production. All clinical isolates used in this study were obtained from denture wearers. It is also possible that *C. albicans* strains may have an inherently higher SAP activity,[23] which is a very relevant aspect of *C. albicans* adaptation to host tissues and virulence. In addition, a further possibility that has to be considered is that perhaps this enzyme is not of fundamental importance for invasion and damage to epithelium tissues. Recent studies evaluated the role of SAP in invasion and damage of oral mucosa by different *Candida* species.[37,42–44] Surprisingly, they found that the inhibition of SAP by the presence of a specific inhibitor, pepstatin A, did not prevent tissue invasion or histological damage induced by yeast cells. Furthermore, in this growth condition, lactate dehydrogenase activity was also not reduced, reinforcing the view that the extent of tissue damage was maintained even when SAP activity was inhibited.[43,44] Thus, the role of SAP activity on the infection process still needs further investigations.

Besides the important role of PL and SAP in the pathogenicity of *C. albicans*, there are available data showing correlations between enzymatic activity and the ability of yeast to adhere to buccal epithelial cells,[20] increased resistance to fluconazole,[20] biofilm formation[26] and denture stomatitis severity.[28] The propensity of *C. albicans* to become a pathogen is a complex process regulated by a dynamic equilibrium between the virulence factors and the immune response of the host. It is possible that a single factor is not the responsible for *Candida* virulence and not all expressed virulence attributes may be necessary for the establishment of an infection. Although *C. albicans* is still the most frequently isolated species from patients with *Candida*, the growing prevalence of non-*albicans* species in diabetics and NDOC is clearly a concern[2,3,12] and their pathogenicity cannot be underestimated. Many of these *Candida* species have also shown the ability to produce extracellular hydrolytic enzymes,[11,18,23–25,27] such as PLs and SAPs. However, there are only a few studies that evaluate a large number of *Candida* spp. isolates from diabetic[11] and non-diabetic patients[23,24,33] suffering from oral candidiasis. Thus, further studies are needed to evaluate the enzymatic activity of biofilms of non-*albicans* clinical isolates of *Candida* and their possible role in the infection process.

Acknowledgement

This work was supported by CNPq - National Counsel of Technological and Scientific Development (grant 501033/2011-4).

Conflict of interest

The authors state that they have no conflict of interest.

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